Role of Gut Microbiota in Morphine-induced Analgesic Tolerance

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Abstract

Prolonged exposure to opioids results in analgesic tolerance, drug overdose, and death. The mechanism underlying morphine analgesic tolerance still remains unresolved. Currently, there are studies focusing on the role of the gut microbiota and its interactions with gut and brain (the microbiota-gut-brain axis) in substance abuse, especially opioid abuse. The microbiota-gut-brain axis is a bidirectional communication between central nervous system and gastrointestinal system. Emerging literature provides evidence that the gut microbiota orchestrates neurogenesis, brain development and function, as well as blood-brain-barrier integrity, and host behavior through vagal afferents, gut hormones, cytokines, and microbial metabolites. Gut dysbiosis disrupts the homeostasis between brain and gut in neurodegenerative disorder, central nervous system injury, and exacerbates disease progression. In opioid abuse subjects, increased comorbidity and behavioral changes are found to be associated with impaired gut integrity and bacterial translocation. We show that morphine analgesic tolerance was significantly attenuated in germ-free (GF) and in pan-antibiotic-treated (ABX) mice. Reconstitution of GF mice with naïve fecal microbiota reinstated morphine analgesic tolerance. We further demonstrated that tolerance was associated with microbial dysbiosis with selective depletion in *Bifidobacteria* and *Lactobacillaeae.* Probiotics, enriched with these bacteria, attenuated analgesic tolerance in morphine-treated mice. These results suggest that probiotics therapy during morphine administration may be a promising, safe, and inexpensive treatment to prolong morphine's efficacy and attenuate analgesic tolerance. We hypothesize a vicious cycle of chronic morphine tolerance: morphine-induced gut dysbiosis leads to gut barrier disruption and bacterial translocation, initiating local gut inflammation through TLR2/4 activation, resulting in the activation of pro-inflammatory cytokines, which drives

morphine tolerance.

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BACKGROUND-LITURATURE REVIEW

1. GUT-BRAIN AXIS

A. Concepts of gut-brain axis

The **gut-brain** axis is a bi-directional neuro-humoral communication system that links gut and brain function (emotional and cognitive centers)(1). The axis involves neural, endocrine and immunological signaling, vagus nerve, and microbial metabolites to integrate the two organs(2). The microbiota has functioned as a key player in the control of this axis. The gut **microbiota** is an ecological community of trillions of commensal, symbiotic and pathogenic microorganisms, including bacteria, archaea, yeast, helminth, parasites, virus, and protozoa(3). The Human Microbiome Project reveals distinct gut microbiome in health and disease. Gram-positive *Firmicutes*, gram-negative *Bacteroidetes*, are the dominant phyla that inhabit the human gut. *Actinobacteria*, *Fusobacteria*, *Proteobacteria* and *Verrucomicrobiota* are present at subdominant levels(4). This complex and dynamic bidirectional relationship of host v.s. microbiota is fragile (**symbiosis**) and can be easily disrupted. When the balance is disturbed, the beneficial and dominating species are underrepresented or outcompeted by hazardous species (**dysbiosis**)(5). The microbiota serves many important functions. It confers food digestion, regulating gut motility, intestinal barrier homeostasis, nutrient absorption, and immune system development, maturation and defense, fat distribution, essential vitamins and amino acids synthesis(2)(6).

B. The gut microbiota is related to human health

Microbiota and their host organisms are mutually co-dependent for survival. Microbiota has been linked with different organs and tissues in the host, including liver,

intestine, cardiovascular, lung, brain, bone and adipose tissue(1). Dysbiosis is associated with various diseases by affecting microbial composition, diversity and metabolites, such as NAFLD/NASH (Non-alcoholic fatty liver disease/ Nonalcoholic steatohepatitis) in liver, inflammatory bowel disease (IBD)/ Crohn's disease in intestine, heart failure, allergic asthma in lung, autism spectrum disorder, stress and stroke in brain, obesity and Type 2 diabetes, systemic lupus erythematosus, atherosclerosis as autoimmune disease(7)(8)(9)(10)(11)(12)(13)(14)(15) (Fig. 1.1).

The gut microbiota is influenced by many aspects: mechanical bowel cleaning, antibiotics, stress-released hormones (catecholamines), vasoactive drugs (norepinephrine), endogenous and exogenous opioids (morphine), enteral feeding restriction, micronutrients insufficiency, operation/ gut manipulation/ resection/ anastomosis, **probiotics** (which contain live beneficial bacteria), **prebiotics** (which contain supplements that promote the growth of specific bacteria), neonatal mode of delivery, breast feeding, environmental exposures early in life(16).

C. Various communication pathways in gut-brain axis

There is increasing evidence that gut microbiota is implicated in the neural system development and neurodegenerative disease(17)(18)(19). Studies comparing GF mice and ABX-treated mice with conventionally raised controls elucidate the role of the gut microbiota in nervous system development and function(20). The majority of gut-brain communication is being facilitated via 4 distinct routes: 1) microbial metabolites; 2) cytokines; 3) vagus afferent; and 4) gut hormones(21) (Fig. 1.2).

1) Microbial metabolites

Gut microbiota produces bioactive molecules, such as SCFA, niacin, urolithins, equol, indole, protocatechuic acid, and 8-prenylnaringenin, that interact with host. Microbiota also stimulates the synthesis and release of neurotransmitters, GABA, acetylcholine, norepinephrine, dopamine, and serotonin(22). These various metabolites cross the intestinal barrier and flow into the circulatory system. They cross the blood brain barrier (BBB) to modulate neurological functions and behaviors. Using capillary electrophoresis mass spectrometry with time-of-flight (CE-TOFMS), different intestinal metabolic profile between GF mice and GF mice transplanted with SPF mouse microbiota was identified(23). The GF mice have reduced levels of metabolites associated with energy metabolism such as pyruvic acid, citric acid, fumaric acid, and malic acid(24). However, GF mice have elevated noradrenaline, dopamine and 5-HT turnover in the striatum compared to SPF mice, accordingly displaying increased motor activity and decreased anxiety(25). Therefore, a comprehensive understanding of microbiota metabolites is critical for clarifying the microbiota-host interactions.

Recent studies investigated the potential of bacterial metabolites to drive host phenotypes and disease outcomes. The levels of 5-HT (serotonin) are found to be significantly lower in the GF mice. However, the administration of *Lactobacillus plantarum PS128* significantly increased serotonin level in the striatum, thus relieving animal's early life stress(26). *Bifidobacterium infantis* has antidepressant properties in rats through changes in the tryptophan/kynurenine pathway(27). It indicates the microbial metabolites from probiotics could improve anxiety-like behaviors. The prebiotics administration altered the mouse gut microbiota, relieved chronic psychosocial stress by increasing acetate and propionate and decreasing butyrate(28). These above examples indicate the constant, life-long interaction of gut microbiota and

human host, which influence neurological function and behavior during development or within health and disease states.

2) Cytokines and regulation of immune cells

Gut microbiota contribute to behavior changes via induction of cytokine secretion. During bacteria or virus infection, the immune system is activated and induces the production of pro-inflammatory cytokines. The cytokines, generally hydrophilic, cross the BBB and deposit into the CNS, leading to a sickness reaction characterized by behavioral symptoms such as fatigue, lassitude, inability to concentrate, irritability, loss of appetite and withdrawal from social interactions(29). In the VPA-induced autism spectrum disorder (ASD) mouse model, the microbial composition was shown to be altered, with increased neutrophil infiltration and intestinal inflammation(30). There is converging evidence showing dysbiosis contributing to an exacerbated neuroinflammatory status leading to dysfunction of brain areas related with mood regulation, learning, and memory. For instance, in non-celiac gluten sensitivity (NCGS), a chronic functional gastrointestinal disorder, gut dysbiosis induces gut inflammation, and abdominal pain. The LPS from pathogenic bacteria activates the systemic inflammation and oxidative stress, and ultimately triggers neuroinflammation and promotes NCGS-induced dementia(31). These studies indicate dysbiosis are crucial drivers of neuropsychiatric complications.

Experimental autoimmune encephalomyelitis (EAE) is attenuated in GF mice with lower levels of proinflammatory cytokines IFN-γ and IL-17A in spinal cord but higher number of $CD4^+CD25^+Foxp3^+$ regulatory T cells. In addition, GF animals failed to stimulate proinflammatory T cell responses. However, GF animals with Segmented

Filamentous Bacteria (SFB) supplement showed EAE development and neuroinflammation(32)(33). Additionally, in the mouse model of middle cerebral artery occlusion (MCAO)-induced ischemic brain injury, mice with the antibiotics amoxicillin and clavulanic acid resistant bacteria (dysbiosis) induced intestinal DCs priming, leading to the expansion of local IL-10 secreting- T_{reg} cells in the small intestine and suppression of effector IL-17^+ γδ T cell function. Less effector T cells traffic from gut to meninges, thus decreasing post-ischemic chemokine (CXCL1 and CXCL2) expression and leukocyte infiltration, reduced infarct volume and thus improving sensorimotor function(34). FMT of brain-injured animals developed significantly larger infarct volumes after cortical lesions in the MCAO stroke model compared with controls that recolonized with sham-surgery microbiota. However, restoring healthy microbiota daily in experimental animals reduced the lesion after large stroke with increased number of $Foxp3^+$ T_{reg} cells(35). While the role of microbiome in influencing CNS immunologic activities is well established, better insight can be gained by identifying the particular bacteria that are associated with behavior such that treatment strategies can be developed.

3) Gut hormones

Gut hormones, in addition to neuropeptides, secreted by enteroendocrine cells in gastrointestinal tract, are found to play a role of neuromodulators in the central and peripheral nervous systems. NPY (neuropeptide Y) regulates cognitive processes. In both GF and antibiotics cocktail mouse models, NPY mRNA expression are prominently increased, and the novel object recognition memory is compromised in these antibiotic-treated mice(36).

Autoantibodies (autoAbs) against appetite-regulating peptides are associated with eating disorders. The National Center for Biotechnology Information (NCBI) database showed that pathogenic micro-organisms such as *E.coli* strains, *Helicobacter pylori, Clostridium tetani, human immunodeficiency virus type 1, C. albicans, A. fumingatus,* and *C.neoformans* displayed a sequence homology of α-MSH. The results imply that autoAbs cross-react with these bacterial components and trigger the eating disorders. Levels of IgA autoAbs against appetite-regulating peptides agouti-related protein, melanin-concentrating hormone, corticotropin-releasing hormone were found decreased in GF rats compared with conventionally raised controls, while the levels of antighrelin IgG were increased. It indicated that the gut microflora control appetite indirectly by regulating the formation of autoantibodies against neuropeptides/peptides involved in this process(37).

4) Vagal

The vagal afferent pathway has emerged as an important means of communicating signals from gut to CNS. Gut microbiota is a source of various biologically active peptides and mediators such as GABA, serotonin, norepinephrine, melatonin, histamine, and acetylcholine(38)(39). Specific bacterial communities have been found to be associated with neurotransmitter synthesis or metabolism. For example, *Lactobacillus spp*. and *Bifidobacterium spp.* produce GABA; *Escherichia spp., Bacillus spp.* and *Saccharomyces spp*. produce noradrenalin; *Candida spp., Streptococcus spp., Escherichia spp.* and *Enterococcus spp.* produce serotonin; *Bacillus spp.* produce dopamine; and *Lactobacillus spp.* produce acetylcholine(40). These neurotransmitters and neuromodulators are released into the blood circulation, cross the BBB and act on vagus nerve to regulate host behavior. For example, acetylcholine release from vagus

nerve plays an immunosuppressive role in inhibiting the release of macrophage TNF-α and attenuating systemic inflammatory responses by binding to acetylcholine-receptor α7 subunit(41).

The presence of certain bacteria in the gastrointestinal tract have an impact on brain function and behavior. For example, mice challenged with subclinical dose of pathogen *Campylobacter jejuni* (*C. jejuni*) exhibited anxiety-like behavior by reducing exploration in open arms of the plus maze(42). Brain activation is observed in the nucleus tractus solitarius (NTS), which is the first entrance of vagal afferents in the brain, indicating the vagus nerve involvement. Similarly, GABA, as main CNS inhibitory neurotransmitter, its concentration and GABA receptor expression are involved in pathogenesis of depression and anxiety. Chronic administration with *L. rhamnosus* induced region-dependent alterations in GABA(B1b) and GABA(Aα2) mRNA in certain brain regions. Thus, *L. rhamnosus* reduced stress-induced corticosterone concentration in plasma and depression- and anxiety-related behavior. However, the effects of *L. rhamnosus* on neurotransmitter and behavioral effects were not found in subdiaphragmatically vagotomized mice(43). The anxiolytic effect of *Bifidobacterium longum NCC3001* on chronic colitis induced anxiety-like behavior was dependent on vagus integrity(44). These studies implicate the vagus system as one of the major communication pathways between gut and brain.

D. Research tools in gut microbiota studies

In research, gnotobiotic animal models were developed to decipher the gut microbiome. **Germ-free** (**GF**) animals are powerful tool to demonstrate direct involvement of components of the gut microbiota in human health and diseases. GF animals selectively

colonized with one or more bacterial species allow researchers to study their effects isolated from other microbes. The **fecal microbiota transplantation** (**FMT**) from human patients into GF mice allow investigators to determine whether dysbiosis associated with a disease is a cause, a contributing factor, or a consequence of the disease state. However, GF mice have numerous immune abnormalities, including underdeveloped lymphoid follicles, fewer and smaller Peyer's patches and mesenteric lymph nodes, defects in antibody production, diminished numbers of T cells and B cells and deficits in cytokine production(45). Therefore, antibiotics-treated mice become an alternative choice. Antibiotics-treated animals are used to investigate effects of disrupting the microbiome in specific life stages of the host and effects of targeting certain groups of bacteria (Gram-positive or Gram-negative).

2. GUT-BRAIN AXIS IN OPIOIDS ABUSE

A. Opioid tolerant patients are at higher risk of sepsis and complications due to morphine's deleterious effects on gut.

Chronic opioid consumption has a lot of deleterious effects. It is associated with higher risk of coronary artery disease, bladder cancer, liver cirrhosis, deep vein thrombosis, asthma and chronic obstructive pulmonary disease(46)(47)(48)(49)(50)(51). Opioid use is also found to associate with a higher risk of gastrointestinal mortalities such as esophageal cancer in a dose dependent manner(52). In opioid users, the increased morbidity was driven largely by infectious complications (surgical site infection, specifically superficial surgical site infection, pneumonia and sepsis)(53)(54)(55). Therefore, opioid users, especially opioid tolerant patients had a significantly longer length of hospital stay and a greater all-cause hospital readmission rate than the non-opioid users(55). As a result, the opioid tolerant population is at risk given the poorer

outcomes and higher health care costs associated with their care(56). One of the explanations is that morphine is associated with impaired bacterial clearance and enhanced susceptibility to infectious organisms(57). Higher circulating morphine levels are observed in patients with sepsis, severe sepsis and septic shock(55). Morphine treatment is suspected to serve as a cofactor in the precipitation of complications by impairing gut barrier integrity, which allows bacterial translocation from the gut lumen into the peritoneal organs and circulatory system(58)(59).

Among all septic patients, both gram-positive and gram-negative bacterial infections were common in opioid-treated patients(55). Fungal infection was more prevalent in opioid-treated patients compared with non-opioid-treated patients as well(55). The most prevalent gram-positive bacteria were *Staphylococcus, Streptococcus*, and *Enterococcus* and the most prevalent gram-negative bacteria were *Escherichia coli*, *Salmonella*, and *Campylobacter*(58)(60). *Candida albicans* was the most prevalent fungus(61). In mice, morphine enhanced *Proteus Mirabilis*, gram-negative bacteria, translocation to liver, spleen and peritoneal cavity(62). Previous data in our lab showed that gram-positive bacteria such as *Enterococcus sp., Staphylococcus sp.,* and *Bacillus sp.* disseminated into MLN, spleen and liver, therefore exacerbating sepsis in a mouse cecal ligation and puncture (CLP) model(58). These findings implicated that morphine-induced expansion of gram-positive bacteria and gram-positive bacterial dissemination inhibits bacterial clearance.

B. Morphine induces gut dysbiosis and enhances the virulence of pathobiome.

We further unraveled that morphine induced a global change in the gut microbiota. A significant shift in gut microbial composition was observed in morphine-treated

animals with a preferential expansion of *Enterococcaceae, Staphylococcaceae, Bacillaceae, Streptococcaceae* and *Erysipelotrichaceae*(60). It implies that the poorer outcomes from opioid-using patients were due to gut microbiota shift to more pathogenic strain. Moreover, comparing intermittent morphine injection with continuous morphine pellet, the intermittent injection model exhibited a more distinct microbial profile with decreased beneficial communities *Lactobacillus* and an expansion of more pathogenic strains such as *Ruminococcus*(63).

Recent studies of C. Acharya found that chronic opioids (most case oxycodone) exhibited a different microbiota profile compared to non-opioid hepatic encephalopathy (HE) patients. Autochthonous taxa (*Lachnospiraceae, Ruminococcaceae,* and *Clostridiales XIV*) were significantly decreased in HE patients on opioids while *Bifidobacterium* was higher. In no-HE patients, opioids use caused decreased *Parasutterella* abundance while increased *Peptostreptococcaceae* abundance. The lower abundance of autochthonous bacteria and *Bacteroidaceae* was correlated with outgrowth of potentially harmful pathogens and longer hospitalizations. The PiCRUST (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) functional metagenomics analysis predicted that opioids-induced microbiota alteration associated with the functions related to metabolism of aromatic amino acids and degradation of potentially beneficial branched-chain amino acids while gut microbiota related to branched-chain amino acid synthesis and bioenergetics processes were more abundant in non-opioid users. In HE patients on opioids, gut microbiota were associated with endotoxin and endotoxin protein synthesis, nitrogen metabolism, motility and degradation of the branched-chain amino acids compared to those with non-opioids HE patients. These opioids-induced dysbiosis partially explained increased IL-6 in serum,

worsened endotoxemia, leading to higher all-cause readmissions in patients with cirrhosis(64).

Additionally, detailed research by John C. Alverdy group showed that opioid (U50,488, a highly selective kappa opioid) increased the virulence of bacteria in critical ill patients. These studies revealed a significant decrease in bacterial diversity with a predominant abundance of *Enterococcus, Staphylococcus,* and *Enterobacteriaceae* and an absence of *Bacteroidetes* in critical ill patients on opioids. In this study, *C. elegans* was used to evaluate survival rate and to test the virulence potential of the bacterial community. When exposure to opioids (50uM of U-50, 488), the bacterial community shifted to a pathogenic phenotype via quorum-sensing signaling systems, thus *C. elegans* survival rate decreased. It is hypothesized that opioids create a stress environment derivative of extracellular phosphate; thus, virulence expression of bacteria is enhanced. Supplementation with Pi-PEG15-20 was protective in opioid treated subjects(65).

Although the mechanisms of morphine-induced gut dysbiosis are elusive, we speculate that morphine abuse and withdrawal commonly cause disruption of neurotransmission in enteric nervous system, gut motility, mucosal immunity, secretion of water, electrolytes, and bile acids in the gastrointestinal tract, resulting in constipation or diarrhea(66)(67). These may contribute to morphine-induced dysbiosis.

C. The mechanisms how gut microbiome affects opioids-induced behavior changes.

The role of the microbiome in opioid-associated behavioral outcomes was tested in several studies using the antibiotic depletion strategy. Gut flora depletion prevented chronic morphine-induced analgesic tolerance by preserving gut integrity, stopping

colonic mucosal disruption, and down-regulating colonic IL-1β expression. It was found that inactivation of tetrodotoxin-resistant (TTX-R) Na^+ channel in nociceptive primary afferent neurons was mitigated when exposed to morphine-tolerant mice colonic supernatant. However, it was prevented by oral vancomycin. These studies indicated that bacterial products, proinflammatory cytokines and other components produced as a consequence of morphine-induced dysbiosis provide an environment for inhibition of TTX-R Na^+ channels inactivation in dorsal root ganglions, which explains the attenuation of morphine tolerance in bacteria depleted condition(68)(69).

Data from our laboratory identified *Enterococcus faecalis (E. faecalis)* as a biomarker of morphine induced dysbiosis. Gavage of *E. faecalis* into mice exacerbated morphine analgesic tolerance(69).

In a more recent study, using antibiotic depletion strategy, mice with depleted microbiota showed no preference for either the cocaine- or saline- paired chamber in conditioned place preference study. It indicated gut microbiota participate in cocaine reward behavior. Additionally, mice with FMT from morphine-treated donors had increased microglial cell body size and activated microglia in both the midbrain and dorsal horn of the spinal cord compared to FMT from saline microbiota transplant. Thus, FMT from morphine-treated mouse disrupted cocaine rewarding pathway(70). Manipulation of gut microbiome without direct exposure to morphine also had an influence on neuroinflammation and opioid-dependent behavior.

To investigate if a specific bacterial profile can serve as biomarker of addiction, Xu et al demonstrated that addiction (heroin, crystal methamphetamine, ephedrine, heroin +

ephedrine and heroin + crystal methamphetamine) was associated with a significant increase in *Thauera, Paracoccus* and *Prevotella* bacterial communities. In addition, functional metagenomics analysis using PiCRUST analysis indicated that these bacterial communities were associated with functions such as translation, DNA replication and repair, and cell growth and death were up-regulated in gut microbiota of substance use disorder subjects. The over-represented pathway might explain the substance induced- dysbiosis exerts functional effects on cell communication, cardiovascular diseases, and circulatory system(71).

Studies have shown that the dynamic interplay between morphine pharmacokinetics and bile acids concentration. Recent studies from Wang et al. show that morphine disrupted bile acid metabolomics profile. The concentration of cholic acid, deoxycholic acid, chenodeoxycholic acid, keto-deoxycholic acid, muricholic acid haven been found decreased in mouse fecal matter after morphine treatment for 3 days(60). Simultaneously, morphine metabolism is also regulated by liver function. In patients who have impaired liver function such as in nonalcoholic steatohepatitis (NASH), the concentration of morphine-6-glucuronide (M-6G) and morphine-3-glucuronide (M-3G) were higher compared to healthy controls(72). It implicates that accumulating M-3G and lower concentration of bile acids provide a deleterious environment for *Bacteroidales* communities such as *Bifidobacteria* to survive in the gut. Strict anaerobes such as *Bacteroides* and *Bifidobacteria* produce β-glucuronidase, which exert an essential role in morphine metabolism. They hydrolyze M6G and M3G, allowing them to be reabsorbed as morphine. Morphine treatment reduced abundance of *Bacteroidales* communities, thus less morphine circulates back into system resulting in escalating morphine consumption, indicating the generation of analgesic tolerance.

These recent observations have indicated that indigenous bacteria do indeed alter aspects of their hosts' neurological function, leading to effects on morphine-induced behavior changes, including tolerance, rewarding, and addition. However, the precise mechanisms of how the gastrointestinal microbiota have an effect on neurological function and behavior remain largely unexploited but are likely complicated and varied.

3. POTENTIAL MECHANISMS OF MORPHINE TOLERANCE

US Food and Drug Administration defined opioid tolerance as the use of greater than or equal to 60 mg of oral morphine equivalents (30 mg of oral oxycodone) daily for 7 days or longer, regardless of long-term opioid use(73). Acute opioid tolerance may develop quickly in opioid-naive patients, leading to higher opioid doses and increased risk of opioid-related overdose death(74). The burden of substance abuse is actually growing, due in part to an alarming increase in the rate of opioid abuse. It has been estimated by NIDA that substance abuse costs $600 billion annually in the U.S., including loss of productivity, healthcare spending, and increase of criminal activities. The rate of deaths from drug overdoses has risen 2-fold from 2002 to 2017. Standard medical therapy including psychotherapy, behavior modification and pharmacotherapy is often not curative, and relapse is common. It is urgent to explore the mechanisms underlying morphine tolerance and to develop potential therapies. Several mechanisms are outlined below.

A. Neuroinflammation contributes to morphine analgesic tolerance

Microglia cells and astrocyte are immune cells in the central nervous system. Chronic morphine administration induces these non-neuronal immunocompetent cells to secrete cytokines such as TNFα, IL-1β, IL-6, CCL2 and CX3CL1(75)(76). These cytokines act

on both glia cells and the pre- or post-synaptic neurons. On microglia cells, the expression of purinergic receptor P2X purinoceptor (P2X7R) and P2X4R, and Toll-like receptor 4 (TLR4) were up-regulated following chronic morphine treatment(77)(78). Concomitantly, at the presynaptic level, chronic morphine treatment enhances excitatory synaptic transmission by release of chemokines, substance P (SP), glutamate and calcitonin gene-related peptide (CGRP)(79). The chronic morphine treatment plus the pro-inflammatory environment activate N-methyl-D-aspartate receptor (NMDAR). The activation of the NMDAR on neuron promotes neurotoxicity and neuron apoptosis through regulating inhibitory neuron transmitter GABA transmission(79). Together, the interaction between neurons and immune cells promotes further neuroimmune activation, enhances neuronal sensitization, and exacerbates OPRM signaling thus impacting morphine analgesic tolerance.

The functions of different cytokines, chemokines, innate immune pattern recognition systems are well-characterized in triggering and maintaining morphine tolerance. Blockade of the formation or activation of proinflammatory cytokines such as IL-6, IL-1β, TNFα, or CX3CL1 attenuates the development of tolerance(80). Morphine tolerance was alleviated by minocycline and CXCR3 inhibitor through blocking the interaction of neuron-microglia(81). CXCL12 neutralizing antibody and AMD3100 (CXCR4 antagonist) preserved morphine analgesic efficacy. Administration of cytokines such as CXCL12 accelerates morphine tolerance(82). Blockade of complimentary factor 5 receptor (C5aR) or tissue plasminogen activator (tPA) or neuronal matrix metalloproteinase 9, or neuronal nitric oxide synthase restored morphine antinociception and inhibited the development of morphine tolerance(83)(84)(85)(86). Anti-inflammatory treatment such as recombinant rat IL-

10 (rrIL-10) and ibudilast, glial cell metabolic inhibitor propentofylline and pentoxifylline, astrocyte activity inhibitor fluorocitrate attenuated the development of morphine tolerance(87)(88)(89)(90). However, the precise mechanisms how neuroinflammation interacts with neuron cells and drives morphine tolerance are not delineated.

B. The role of TLR4 and TLR2 in morphine tolerance

The TLR4 signaling pathway is an important contributor to the development of tolerance following continued exposure to opioids. Morphine-3-glucuronide (M3G), one of the major metabolites of morphine, activates TLR4, causes subsequent microglial proinflammatory activation and releases IL-1β. Moreover, TLR4/MD2 complex, followed by MyD88 pathway, drives morphine-induced proinflammatory cytokines IL-1β and TNF-α(91).

Furthermore, ATP-sensitive potassium channel regulates HSP70 release by neuron cells. The HSP70 activates TLR4 signaling pathway on microglia, induces NLRP3 inflammasome activation, and promotes the formation of mature IL-1β by activating caspase-1, thus contributing to morphine tolerance(92). As a result, morphine tolerance was observed to attenuate both in TLR4KO mice and following administration of K_{APT} blocker, glibenclamide(92).

However, the role of TLR4 signaling pathways in morphine analgesia is controversial. Wang et al. showed that both TLR4KO and MyD88KO (the critical downstream component) mice exhibited potentiated acute morphine analgesia(93). However, in a separate study, in contrast to the above described studies, Liu et al. showed that only

TLR4KO mice, but not MyD88KO mice, have an protective effect on chronic morphine tolerance(94). It implies that MyD88 dependent pathway participates in the generation and maintenance of acute not chronic morphine tolerance. However, due to limited studies, the role of MyD88 in chronic morphine tolerance needs to be further investigated.

The interaction between OPRM1 and TLR2/4 signaling pathways remains to be elucidated. TLR2 and TLR4 are involved in pain hypersensitivity and morphine tolerance. For instance, HKLM (TLR2 agonist) induced- lasting tactile allodynia in WT mice was prevented in TLR2KO mice(95). In addition, primary glial cells from TLR2KO mice could not be activated as WT mice by damaged sensory neuron. Thus, the increased sensitivity to both mechanical and thermal stimuli observed in WT mice was attenuated in TLR2KO mice. Therefore, TLR2 is essential to produce pain-mediating pro-inflammatory cytokines for nerve injury-induced spinal cord glial cell activation(96). Moreover, microglia in NAc had a higher expression of TLR2 after chronic morphine treatment. Morphine-induced microglia activation and proinflammatory cytokines were markedly inhibited in the TLR2KO mice. Therefore, the behavioral signs of morphine withdrawal were attenuated in TLR2KO mice, which indicates that TLR2 plays an important role in response to morphine dependence(97). In vitro study indicated that overexpression of TLR2 in HEK293 cells caused cell apoptosis after morphine treatment. However, blockage of TLR2 downstream target MyD88 or blockage of OPRM downstream β-arrestin 2 attenuated morphine-induced cell death(98). In addition, chronic morphine treatment induced decrease of anti-apoptotic Bcl-2 gene expression and increase of pro-apoptotic Bax gene expression in cortex and lumbar spinal cord. Donepezil attenuated morphine-induced tolerance by

suppressing morphine-induced TLR2 and TLR4 expression up-regulation(99). Although there is limited report of the critical role of TLR2 in morphine tolerance currently, inhibition of TLR2 may find application in the development of novel therapies to treat opioid tolerance, dependence and addiction.

C. Signaling pathways in morphine tolerance

Acute morphine administration activates opioid receptors. Dissociation of $G_{i/o}$ from OPRM inhibits the activity of adenylyl cyclase (AC) of cAMP signaling, augments K^+ conductance and inhibits voltage gated Ca^{2+} channel function, which subsequently inhibits synaptic activity by suppressing the release of the excitatory neurotransmitters such as acetylcholine, serotonin, nitric oxide and intestinal peptide(100). However, tolerance development can be due to opioid-receptor changes (decreased activation, receptor desensitization and endocytosis) that occur after prolonged exposure to opioids. Chronic morphine treatment mediates G protein coupling by MOR switching from $G_{i/o}$ to G_s protein and stimulates adenylyl cyclase II and IV by $G_{\beta\gamma}$ dimer. Opioid tolerance activates the intracellular cAMP signaling pathway. The increase in cAMP levels results in the activation of cAMP-dependent protein kinase (PKA). Morphine-induced changes further lead to the activation of transcriptional factor Ca^{2+}/cAMP response element binding protein (CREB), which may be important to the development of tolerance. The activation of $G_{i/o}$ βγ subunit induces inositol lipid hydrolysis and produces IP_3 and diacylglycerol (DAG), which consequently lead to release stores of intracellular Ca^{2+}. It further activates the mitogen-activated protein (MAP) kinase, PKA and PKC(101)(102). Activated opioid receptor is phosphorylated by a G protein coupled receptor kinase (GRK), which recruits β-arrestin complex. The activated opioid receptor is desensitized and internalized. It is either recycled to the cell surface

or targeted for lysosomal degradation. Although chronic morphine treatment does not cause opioid receptor internalization(103), tolerance was attenuated by intrathecal β-arrestin 2 small interfering RNA in rats(45). Additionally, one study has shown that morphine-induced OPRM internalization was detected in the presence of an inhibitor of conventional PKC isoforms(104). There is another study implying that PKC-mediated desensitization of OPRM, suppressing cAMP-inhibition, thus exacerbating morphine tolerance(105). Additionally, intrathecal injection of siRNA against PKA blocked morphine tolerance by inhibiting morphine augmentation on calcitonin gene related peptide (CGRP) synthesis and release in primary sensory neurons(106). These studies help us gain insight into the mechanisms how interaction of different signaling pathways plays a key role in morphine tolerance. However, there is still no clear consensus on how chronic opioid use leads to analgesic tolerance.

D. Role of Blood-Brain-Barrier in morphine tolerance.

On the blood-brain-barrier (BBB), several ATP-binding cassette (ABC) transporters participate in the efflux of xenobiotics such as opioids from the CNS into bloodstream. It controls the brain concentration of the drugs and their effects on CNS and is responsible for the efflux of opioids. Chronic morphine treatment in rats has shown to have increased expression of P-glycoprotein (P-gp), thus implicating it in morphine tolerance. It has been reported that high level of glutamate induced by morphine tolerance activates NMDA receptor to up-regulate P-gp through COX-2. Another study demonstrated that chronic morphine exposure activates TLR4 with subsequent release of TNFα. TNFα binds to its receptors on endothelial cells and up-regulates P-gp expression through NF-kB(107).

However, whether chronic morphine modulates BBB integrity is controversial. Oishi et al. mentioned acute morphine resulted in increased brain permeability to sodium fluorescein in mice through activation of H_2-receptors by neuronal histamine and muscarinic receptor by acetylcholine(108). Another study indicated that morphine withdrawal not morphine tolerance resulted in damage of BBB integrity. Microglia cells, which were activated by morphine withdrawal, mediated oxidative stress response and promoted BBB permeability(109).

4. SUMMARY

As delineated above, growing evidence from animals and human studies has shown that opioids can have a major impact on composition and function of gut microbiota. What is most intriguing from a treatment perspective is that modification of gut bacteria (antibiotics and FMT) can reverse the abnormalities of behavior changes related with substance abuse. It implies that opioid abuse-induced dysbiosis influences psychopathology by affecting gut permeability, microbial metabolites concentration, local inflammation and neuroinflammation, the related receptor expression and activation in CNS, and drug pharmacokinetics. The gut microbiota manipulation (prebiotics, probiotics, antibiotics, FMT) hold the greatest promise for novel treatments for opioids abuse. Basic research clinical trials are required to examine what is more efficacious to yield new insights into the role of gut-brain axis in opioid abuse. Although the microbiota-gut-brain axis is attractive in the field of substance abuse, this research is still in its infancy.

Morphine tolerance may be multifactorial involving interaction between neurons, non-neuronal immune cells and endothelial cells, neuroinflammation, activation of TLR and

alteration of OPRM1 signaling pathway (Fig 1.3). Although it has been well demonstrated that morphine treatment results in gut dysbiosis, impaired gut barrier integrity and bacterial translocation, it is unclear how morphine-induced gut pathologies exert influence on neuroinflammation and related behavior changes. We hypothesize that chronic morphine treatment induces dysbiosis and triggers gut inflammation. The cytokines generate from local inflammation cross BBB and mediate their effects directly in the CNS, which in turn modulate analgesic tolerance.

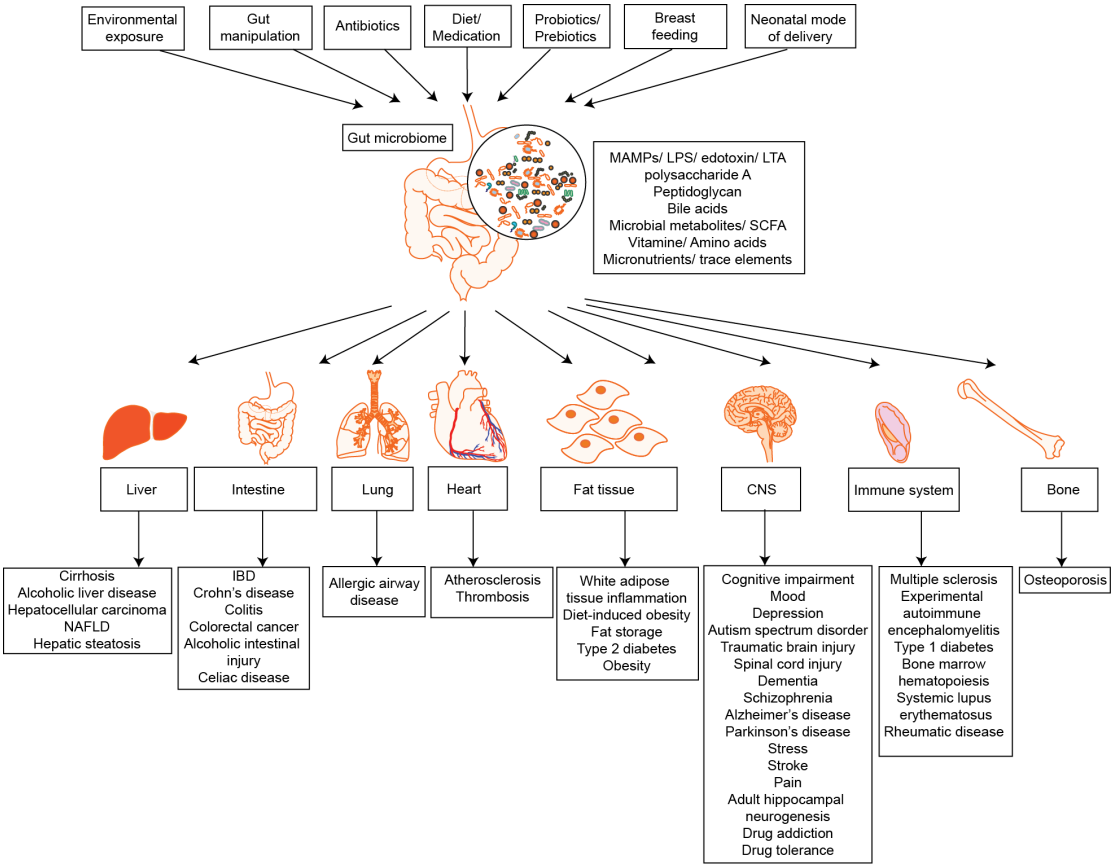


Figure 1.1 Gut microbiota and host have a bi-directional communication. The host diet, medication and living habits are associated with microbiota composition and diversity. The gut microbiota transform dietary molecules and environmental factors into metabolites and signaling molecules to regulate the host's health. The gut microbiota plays a key role in various diseases. It affects different organs in human body as indicated above.

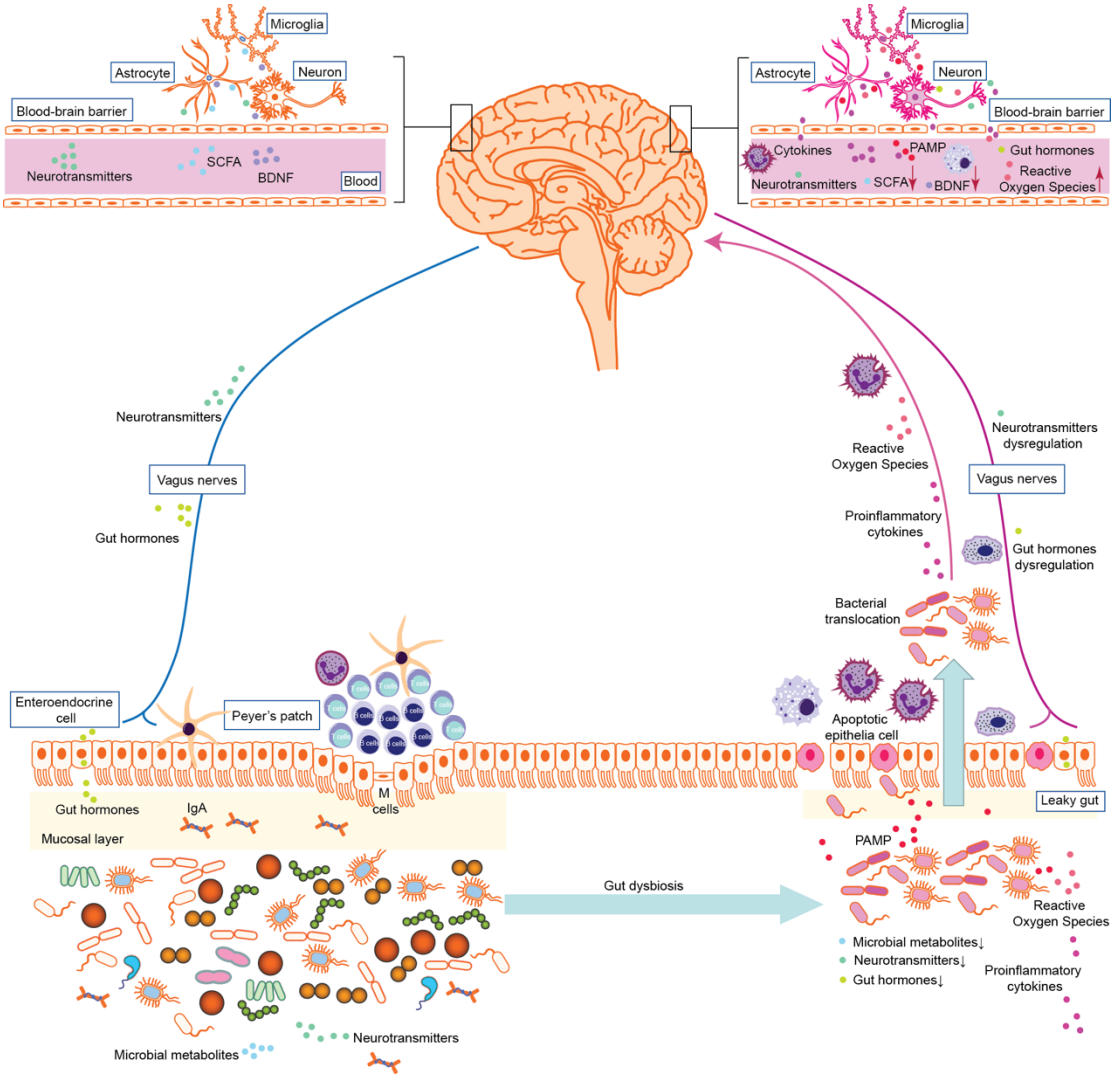


Figure 1.2 Interactions between the gut microbiota, peripheral system and CNS are essential for the maintenance of host health. The microbial-derived products (microbial metabolites) and metabolic products (SCFA, gut hormones) transport into host system through bloodstream and vagus system to induced CNS development and maintain brain homeostasis. In the disease model, when gut homeostasis is disrupted, the pathogenic bacteria outnumber beneficial bacteria. The gut integrity is damaged, and bacterial translocation occurs. The translocated bacteria and pathogen associated molecular patterns (PAMPs) activate peripheral immune cells to produce proinflammatory cytokines and chemokines. The cytokines and chemokines cross the blood brain barrier and exert deleterious effects directly on CNS. Simultaneously, the imbalance between beneficial bacteria and pathogenic bacteria results in abnormal

secretion of microbial metabolites, neurotransmitters and gut hormones, which have detrimental effects on BBB integrity, neurons, astrocytes and microglia, therefore, driving neurological disorders.

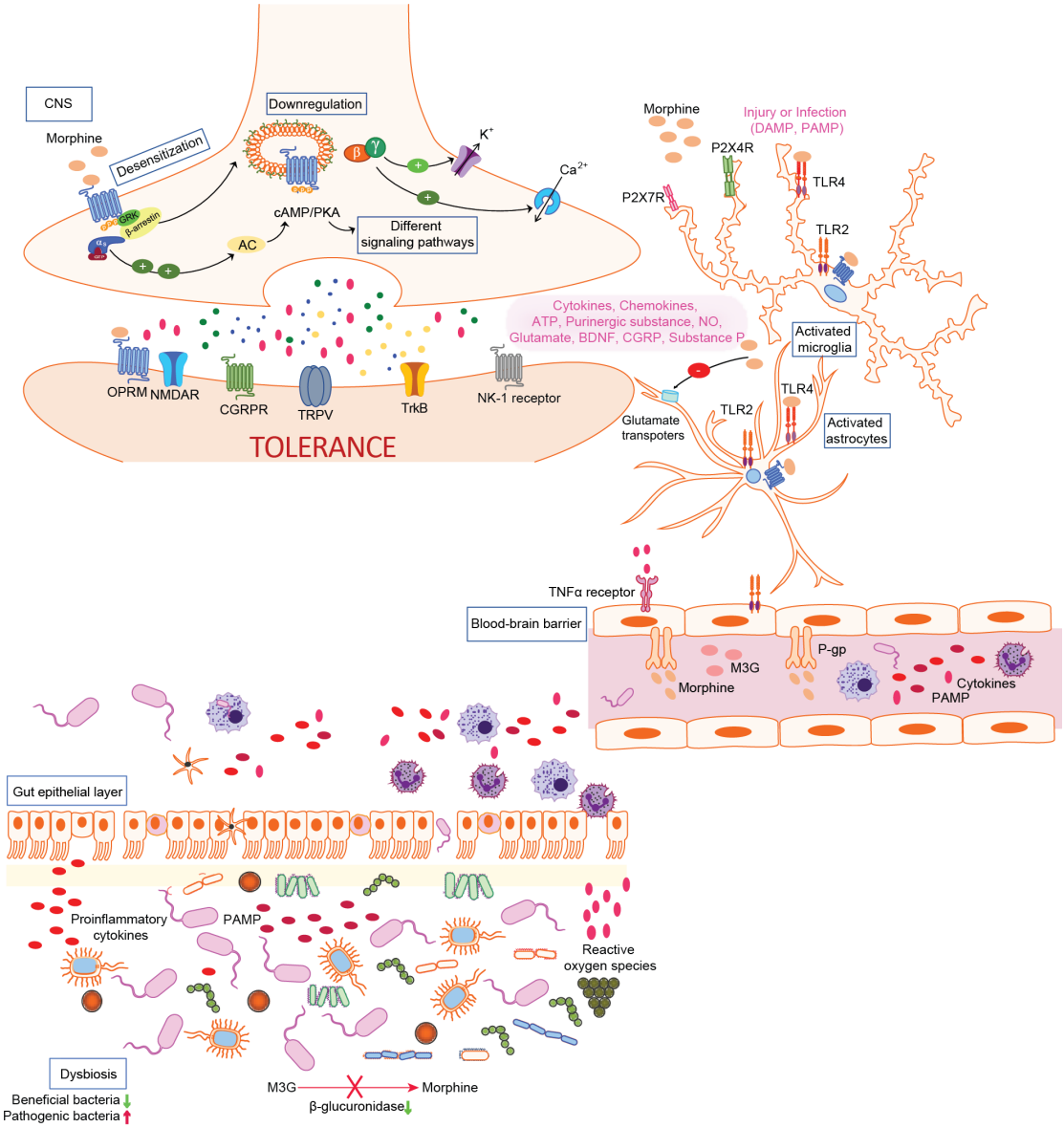


Figure 1.3 Mechanisms of morphine analgesic tolerance. 1. In opioid long-term treatment, the phosphorylated receptors are desensitized and retained on the membrane. 2. β-arrestin binding to the receptor leads to internalization, degradation, and down-regulation receptor on membrane. 3. Chronic morphine treatment super-activates adenylate cyclase (AC) activity and activates PKA/MAPK signaling pathways. 4. The activity of voltage-gated calcium channels are up-regulated to increase intracellular Ca^{2+}, thus activating calmodulin kinase and PKC. These activated signaling pathways can modulate multiple targets including neurotransmitters, cytokines and chemokines, and their receptors, and related other proteins, thus contributing to analgesic tolerance.

5. Chronic morphine treatment activates N-methyl-d-aspartate (NMDA) receptors and suppresses glutamate transport on astrocytes to increase synaptic glutamate levels. 6. Non-neuronal cells such as microglia and astrocytes release inflammatory mediators through activation of toll-like receptors (TLRs). The released proinflammatory substances (inflammasomes, ATP, and calcitonin gene related peptide (CGRP), substance P, brain-derived neurotropic factor (BDNF)) sensitize the pre- and postsynaptic central neurons, contributing to tolerance. 7. Morphine directly binds to TLR4 to activate microglia and astrocytes. 8. TNF-α binds to endothelia of BBB to up-regulate p-glycoprotein (P-gp) expression, thus exporting morphine molecules out of CNS to generate tolerance. 9. Chronic morphine induces gut dysbiosis, causes bacterial translocation and initiates local inflammation. The microbial components and pathogen associated molecular patterns (PAMP) circulate into CNS, activate non-neuron immune cells, result in increased expression of pro-inflammatory cytokines, and contribute to morphine tolerance. 10. Chronic morphine treatment depletes the beneficial bacteria with β-glucuronidase activity. The morphine metabolite morphine-3-glucuronide accumulates in the gut. Therefore, morphine could not recirculate back through enterohepatic recirculation. More morphine is needed to maintain the same analgesic effect.

CHAPTER 2

GUT MICROBIOME CONTRIBUTES TO MORPHINE TOLERANCE

INTRODUCTION

Opioids are the gold standard for the management of moderate to severe pain(110). In spite of their high efficacy, the clinical use of opioids is limited because of co-morbidities associated with their prolonged use(111). The last decade has seen a significant rise in opioid use in the United States resulting in a subset of the population developing opioid tolerance(112)(113). Chronic and repeated opioid use leads to the rapid onset of analgesic tolerance; however, the pharmacokinetics of tolerance to peripheral receptors develop slowly leading to respiratory depression, immune modulation, nausea and decreased gastrointestinal motility with escalating doses of opioids(114). Thus, chronic opioid use is associated with poorer outcomes, longer lengths of hospital stay, higher readmission rates and higher health care costs(54)(56). Therefore, it is imperative that we understand the mechanisms underlying the co-morbidities associated with chronic opioid use and delineate specific protocols for the care of chronic pain patients that are on opioids. However, the precise mechanisms underlying morphine tolerance remain unresolved. Recent studies have shown that the gut microbiota plays a crucial and dynamic role in immune response and neuronal function(115). Gut homeostasis confers health benefits, and any disruption resulting in alteration in beneficial bacteria can negatively influence the health and wellbeing of an individual(1)(116). Toll-like receptors (TLRs) recognize a variety of microbial components and allow the innate immune system to sense and react to the altered microbiota, hence playing a central role in the interaction between host and microbiota(117).

Our recent studies have shown that chronic opioid use in animal models is associated with altered gut microbiota, with an expansion of *Firmicutes* and a decrease of *Bacteroidetes* (60)(118). These findings are supported by studies in patients with substance use disorder who demonstrate altered composition and diversity of gut microbiome distinct from healthy controls, thus implicating the gut-brain axis in morphine analgesic tolerance (64)(71)(119). In the present studies, we used multiple murine models to demonstrate the essential role of the gut microbiota in morphine analgesic tolerance. These studies suggest that strategies can be developed to alter the gut microbiota to prolong the effectiveness of morphine and prevent analgesic tolerance.

MATERIALS AND METHODS

Experimental animals

Pathogen-free C57BL/6J mice were obtained from the Charles River Laboratories. All animals were housed in a specific-pathogen-free facility. All procedures were approved by Institutional Animal Care and Use Committee (IACUC) at the University of Miami. All mice (C57BL/6, TLR2KO, TLR4KO, and IL-6KO) were housed 3-5 per cage in SPF conditions and maintained on a 12-hour light/dark cycle in a constant temperature (20–22°C) and humidity (45–55%) with ad libitum access to food and water. GF mice were maintained in CBC flexible film isolators with sterile food and water.

Animal treatment:

Morphine sulfate (National Institutes of Health [NIH]/National Institute on Drug Abuse [NIDA], Bethesda, MD) was dissolved in sterile saline. Mice received constant dose of 15mg/kg or escalating doses of (5, 10, 15, 20, 25, 30, 35, 40mg/kg) morphine injection b.i.d. On the ninth day, 25mg/kg morphine sulfate was used to test the anti-nociceptive

response. According to established protocol, the non-absorbable pan-antibiotics cocktail (ABX) was prepared every day freshly in drinking water(120). The ABX treatment was given 7-10 days before morphine treatment and continued throughout the following eight days of morphine treatment and behavioral measurement.

The mice were administrated with 5 g/L of streptomycin sulphate in the drinking water for 2 days and switched to normal drinking water for 24 hours before *E. faecalis* (EF) inoculation by oral gavage. The spectinomycin sulphate selective *E. faecalis* were diluted up to the concentration of 2 x 10^{10}/mL in phosphate buffered saline (PBS). Each mouse was administered with 200μL spectinomycin solution by oral gavage daily. After 48 hours post gavage, the mice were treated with 250 mg/L spectinomycin sulfate (to prevent overgrowth of pathogenic gram-negative bacteria) in the drinking water during the behavior study. To maintain the population of *E. faecalis* in the mouse gut, mice were administered the same dose of *E. faecalis* and the same dose of spectinomycin sulphate by oral gavage daily during the behavior experiment for 8 days.

Mice were orally gavaged with freshly prepared 5x10^{10} CFU of the probiotics VSL#3 in 200μL water daily for 21 days before morphine treatment and maintained throughout the following eight days(121).

Fecal Matter Transplantation (FMT):

Twenty donor mice (10 mice for each batch) were injected with either morphine or saline b.i.d. as previously described. Their fecal contents were collected and pooled after sacrifice. The fecal content was processed according to the established protocol with modification(118). Briefly, 200mg of the fecal extract was suspended in 1mL

sterile PBS, filtered through 70μM cell strainer, and centrifuged at 6000Xg for 20min. About 10^{10} CFU/mL fecal bacteria were suspended in 6% NaHCO3 buffer with 20% sucrose. Each recipient mouse was treated with ABX as previously described and then orally gavaged with 200μL of freshly prepared fecal suspension on seven consecutive days before any downstream experiment or analysis.

Behavioral study:

The thermal nociceptive thresholds were assessed by both tail flick and hot plate assays as reference(122). The hot plate and tail flick analgesic responses were calculated as the percentage the maximum possible effect (%MPE). %MPE = (post-drug latency – pre-drug latency)/ (cut-off – pre-drug latency) × 100% for tail flick analgesia and hot plate.

16S rRNA gene sequencing:

Contents of ileum were collected under aseptic conditions from all mice after sacrificing. Sequencing and bioinformatics were performed by the University of Minnesota Genomic Center, MN, United States, and Microbiome Insights, Vancouver, BC, Canada. DNA was isolated using DNeasy PowerSoil® kits (Qiagen, Germantown, Maryland) modified to include a bead-beating step. At University of Minnesota, after DNA isolation, 16S ribosomal DNA hypervariable regions V5 and V6 were polymerase chain reaction amplified using primers with the V5F RGGATTAGATACCC and V6R CGACRRCCATGCANCACCT gene-specific sequences, Illumina adaptors, and molecular barcodes as described to produce 427 base pair (bp) amplicons. Samples were sequenced on an Illumina MiSeq (Illumina, San Diego, California) using MiSeq 600 cycle v3 kit(123). In Microbiome Insights, the V4 region was amplified with

adapter-barcode-pad/linker-16S primer as shown below: AATGATACGGCGACCACCGAGTCTACACCTACTATATATGGTAATTGTGT GCCAGCMGCCGCGGTAA and CAAGCAGAAGACGGCATACGAGATAACTCTCGAGTCAGTCAGCCGGACT ACHVGGGTWTCTAAT. Samples were sequenced by Illumina MiSeq.

Metagenomic data analysis:

Primer sequences were removed from raw sequence reads and low-quality bases (Phred score<20) were trimmed from 3' end using cutadapt(124). Microbial taxonomy assignment abundance quantification was analyzed with Greengenes database (ver.08/13) using dada2 pipeline. Microbial diversity between samples (β-diversity) was quantified by Bray-Curtis dissimilarity using R package "vegan"(125). The dissimilarity between pairs of treatment groups was assessed using permutation multivariate analysis of ANOVA (PERMANOVA) adjusting for the batch difference, and the significance of pair-wise comparison was adjusted for multiple comparison using Bonferroni correction. Individual differential taxa were identified accounting for batch difference using DESeq2 with significance determined based on a false discovery rate of 0.05 (FDR)(126). The raw sequencing data that support the findings of this study have been submitted to Sequence Read Archive (SRA) of the National Center for Biotechnology Information under accession number PRJNA531200.

RESULTS

1. *Morphine treatment induced distinct gut microbiota community.*

To determine if microbial dysbiosis contributed to morphine analgesic tolerance, mice were treated with repeated morphine injections to induce analgesic tolerance. On day

8, small intestinal fecal contents were collected from WT morphine-tolerant mice and their corresponding saline-treated controls. Fecal DNA was extracted and subjected to 16S ribosomal RNA sequencing. β-diversity analysis revealed a distinct clustering of the bacterial communities in the morphine-tolerant animals compared to saline-treated mice (p=0.00256, Permutation ANOVA test with Bonferroni correction) (Fig. 2.1).

2. *The Bifidobacteriaceae and Lactobacillaceae were reduced in morphine tolerant rodents.*

Further analysis revealed a reduction in the relative abundance of *Actinobacteria* at the phylum level in morphine-tolerant animals (Fig. 2.2A and Fig. 2.2B); Furthermore, morphine-tolerant animals displayed an expansion of *Erysipelotrichaceae* (Fig. 2.2C), *Peptostreptococcaceae* (Fig. 2.2D), Dehalobacteriaceae (Fig. 2.2E) and *Ruminococcaceae* (Fig. 2.2F), and a reduction in *Caulobacteraceae* (Fig. 2.2G), *Comamonadaceae* (Fig. 2.2H), *Coriobacteriaceae* (Fig. 2.2I) and *Moraxellaceae* (Fig. 2.2J) at family level. At genus level, morphine-tolerant animals exhibited increased *Allobaculum* (Fig. 2.2M) and *Oscillospira* (Fig. 2.2N) and decreased *[Ruminococcus]* (Fig. 2.2O), *Adlercreutzia* (Fig. 2.2P), *Acinetobacter* (Fig. 2.2Q), *Brevundimonas* (Fig. 2.2R), *Turicibacter* (Fig. 2.2S), and *Delftia* (Fig. 2.2T). Notably, *Bifidobacteriaceae* (Fig. 2.2K) and *Lactobacillaceae* (Fig. 2.2L) at the family level and *Bifidobacterium* (Fig. 2.2U) and *Lactobacillus* (Fig. 2.2V) at the genus level were diminished in morphine-tolerant rodents.

3. *Morphine tolerance was attenuated in germ-free mice.*

To initially establish the role of the gut microbiota in morphine tolerance, GF mice and conventionally raised, specific-pathogen-free mice (SPF) were subjected to a well-

established tolerance regimen wherein mice were treated with either saline or repeated escalating doses of morphine for 8 days. Interestingly, morphine-treated GF mice displayed less analgesic tolerance in both tail flick and hot plate tests than SPF mice, 75% and 25% maximum possible effect (%MPE), respectively (Fig. 2.3 A and B).

4. *Morphine tolerance was recapitulated after germ-free mice receive naïve microbiota*

After GF mice that have undergone FMT with samples obtained from SPF naïve mice. These mice showed significant analgesic tolerance similar to SPF animals treated with morphine (Fig. 2.4 A and B). These results clearly establish the role of gut microbiota in morphine analgesic tolerance.

5. *Morphine tolerance was attenuated in pan-antibiotics-treated mice.*

To further investigate if gut microbial dysbiosis contributed to morphine tolerance, SPF mice were administered a cocktail of antibiotics in their drinking water for a week to deplete the gut microbiota and then treated with escalating doses of morphine for 8 days. Pan-antibiotics (ABX) treatment was maintained during the whole duration of morphine treatment. Results from these studies show that ABX markedly reduced gut bacteria (Fig. 2.5 A). ABX and SPF mice were treated with repeated escalating doses of morphine. The morphine-treated ABX mice showed significantly attenuated analgesic tolerance with 64 %MPE at day 8 in both tail flick (Fig. 2.5 B) and hot plate assays (Fig. 2.5 C). 8 days. In an additional study, a fixed morphine dose was administered to ABX and SPF control mice twice daily for 8 days. The ABX mice maintained a significantly higher antinociceptive efficacy from day 4 by tail flick (Fig. 2.5 D) and from day 5 by hot plate (Fig. 2.5 E) compared to non-antibiotic-treated mice.

6. Morphine tolerance was mediated by FMT from morphine-treated mice.

To further establish the role of gut microbiota in analgesic tolerance, SPF mice treated with ABX were gavaged with microbiota harvested from either saline- or morphine-treated mice. Mice reconstituted with morphine-tolerant mouse microbiota and then treated with morphine showed exacerbated analgesic tolerance following the same daily injection doses. In contrast, mice that were reconstituted with saline-treated mouse microbiota displayed less analgesic tolerance (Fig. 2.6 A and B). To validate effective reconstitution, stool samples were collected from recipient mice and subjected to 16S ribosomal RNA sequencing. The microbiome of the recipient animals was similar to their donor profile in principal coordinates analysis (PCoA) clustering (Fig. 2.6 C). In summary, the data from these studies clearly support a role for gut microbiota in morphine-induced analgesic tolerance.

7. Morphine tolerance was exacerbated by pathogenic bacterial infection.

Previously our lab demonstrated a significant expansion of *Enterococcus faecalis* in morphine-treated mice(60). In clinical studies, prescription opioids are associated with higher *Enterococcus* infection in septic patients(55). Therefore, we gavaged *Enterococcus faecalis* into mice to delineate the function of pathogenic bacteria to morphine tolerance. The mice infected with *Enterococcus faecalis* displayed more severe analgesic tolerance to morphine (Fig. 2.7 A, B) compared with morphine treated mice.

8. Dysbiosis induced by morphine-tolerance was attenuated by VSL#3 probiotics.

Our previous microbial analyses showed that the relative abundance in the Operational Taxonomic Unit (OTUs) representing *Lactobacillaceae* and *Bifidobacteriaceae* were significantly reduced in morphine-tolerant animals. To investigate whether supplementation with these beneficial bacteria attenuated morphine tolerance, the mice were gavaged with probiotics VSL#3 21 days before morphine treatment. Measured by β-diversity, probiotics pre-treatment decreased morphine-induced microbial alterations, indicating that VSL#3 probiotics restored partial gut microbial components. (Fig. 2.8 A) ($p=0.00096$ for Water+Morphine and Water+Saline, $p=0.00012$ for Water+Morphine and VSL#3+Saline, $p=0.0024$ for VSL#3+Morphine and Water+Saline, $p=0.02592$ for VSL#3+Morphine and VSL#3+Saline). At phylum level, morphine-induced reduction of Actinobacteria were supplied by VSL#3 (Fig. 2.8 B). Notably, bacterial communities (*Bifidobacteriaceae*, and *Lactobacillaceae* at family level, and *Bifidobacterium*, *[Ruminococcus]*, and *Lactobacillus* at genus level) that were significantly reduced in relative abundance in morphine-tolerant animals were substantially restored compared to saline control sample. *Peptostreptococcaceae*, *Prevotellaceae*, *Erysipelotrichaceae* and *Dehalobacteriaceae* at family level, *Prevotella*, *Allobaculum*, *Dehalobacerium* at genus level, which were found significantly increased in morphine-tolerant mice, were decreased by probiotics supplementation (Fig. 2.8 C and D).

9. *Morphine tolerance was alleviated after gut microbiota was restored by VSL#3 probiotics.*

After the mice were treated with probiotics VSL#3 for 21 days, the mice were treated with either escalating dose or constant dose of morphine. A dramatic decrease in morphine antinociceptive tolerance with VSL#3 pre-treatment was found compared to

sham mice following escalated morphine treatment in both tail flick (%MPE 34.74 vs 54.23) and hot plate (%MPE 30.04 vs 51.09) tests (Fig. 2.9 A and B). Similarly, probiotics pre-treatment alleviated morphine tolerance after 15mg/kg constant doses of morphine treatment (Fig. 2.9 C and D).

10. The morphine-induced gut dysbiosis was prevented in TLR2KO and TLR4KO mice

After the TLR2KO and TLR4KO mice received the same doses of saline or morphine as WT mice, small intestinal fecal contents were collected as WT morphine-tolerant mice and their saline-treated controls. We found no significant difference in the β-diversity matrix comparing morphine and saline-treated animals ($p=0.21668$, Fig. 2.10 A, $p=0.1833$, Fig. 2.10 B). The differential bacterial profile that we observed in WT mice was not observed either in TLR4KO and TLR2KO mice. Furthermore, TLR2KO mice shows less bacterial profile changes compared to TLR4KO mice after chronic morphine administration. These data imply that the microbiota of TLR2KO and TLR4KO are very stable and resist to any change as a consequence of morphine treatment (Fig. 2.10 C, D and E).

11. Morphine tolerance was attenuated in TLR2KO and TLR4KO mice

To further delineate the roles of TLR2 and TLR4 in morphine analgesic tolerance, TLR2 and TLR4 Knockout (TLR2KO and TLR4KO) mice were treated with repeated injections of morphine (either with escalated or fixed doses). We found that morphine-induced analgesic tolerance was partially attenuated in both TLR2KO and TLR4KO mice using either tail flick (Fig. 2.11 A) or hot plate (Fig. 2.11 B) assays in escalating dose model and constant dose model (Fig. 2.11 C and D). Although morphine-induced

analgesic tolerance was modulated by both TLR2 and TLR4, it was more significantly attenuated in TLR2KO mice. This suggests a more important role for TLR2 in morphine analgesic tolerance.

12. Morphine-induced gut dysbiosis was attenuated in IL6KO mice

As previous literature indicated that proinflammatory cytokines play a key factor in morphine analgesic tolerance. Here we used IL-6KO mice to detect whether IL-6 have an effect on gut dysbiosis in the morphine-tolerant model. The IL-6KO mice did not exhibit a significantly distinct bacterial profile after chronic morphine treatment as WT mice (IL6KO_Morphine vs IL6KO_Saline, p= 0.84244, Fig. 2.12 A, B). Furthermore, the dramatic accumulation of *Verrucomicrobiaceae*, *S24-7*, and *Clostridiaceae* at family level, *Akkermansia* and *Clostridium* at genus level in morphine tolerant mice were not observed in IL-6KO mice. Notably, the decrease of *Lactobacillaceae* and *Bifidobacteriaceae* at family level and *Lactobacillus* and *Bifidobacterium* at genus level in WT tolerant mice were not observed in IL-6KO morphine treated mice (Fig. 2.12 C, D). Taken together with previous TLR2KO and TLR4KO microbial profiles, it implies that immune system plays a key role in maintaining bacterial habitat in the gut. It is speculated that IL-6 proinflammatory cytokine induced by chronic morphine treatment makes a deleterious environment for beneficial bacteria such as *Lactobacillaceae* and *Bifidobacteriaceae* to survive and proliferate.

13. Morphine-induced tolerance was alleviated in IL6KO mice

Since morphine-tolerant animals showed a significant increase in IL-6 protein levels, we hypothesized that IL-6 may be one of the mediators of morphine tolerance. To test this hypothesis, IL-6 Knockout (IL-6KO) mice were subjected to repeated morphine

injections to induce analgesic tolerance. Analgesic tolerance for both tail flick (30.23 vs 42.82 %MPE) and hot plate assays (23.44 vs 35.89 %MPE) (Fig. 2.13 A, B, C and D) were only partially attenuated in these mice indicating that other proinflammatory cytokines may also contribute to modulating analgesic tolerance.

DISCUSSION

In previous and current studies, we showed the adverse effects of morphine-induced dysbiosis. The modification of gut microbiome implicates the mediators and consequences of the communication between gut and brain. Our findings identify that gut microbiome plays a causative role through initiating inflammation by TLR2 and TLR4 activation, resulting in morphine-induced analgesic tolerance. We used ABX and GF mice to prove that elimination of gut microflora retained analgesic efficacy, and the morphine tolerance restored after recolonizing gut microbiota. After replenishing the missing gut bacteria, especially *Bifidobacteriaceae* and *Lactobacillaceae* by VSL#3 probiotics, we found that morphine analgesic tolerance was attenuated. In addition, morphine analgesic tolerance was exacerbated in pathogenic *Enterococcus faecalis* infection model. Taken together, these findings implicated the fundamental role of microbiota in the opioid-induced analgesic tolerance.

There are now a large and accumulating body of evidence demonstrating that gut bacteria have the capacity to regulate CNS homeostasis and have given rise to the concept of the microbiota-gut-brain axis which relays a variety of interaction between the CNS and the gastrointestinal tract. However, the precise details of the mechanisms are not fully understood. In morphine tolerant mice, at family level, *Peptostreptococcaceae*, *Erysipelotrichaceae*, and *Dehalobacteriaceae* were found

more abundant in morphine tolerant mice. Bajaj group showed that the opioid users had higher endotoxemia. Moreover, IL-6 level and *Peptostreptococcaceae* were found increased in these chronic opioid users(48). Other studies implied that *Peptostreptococcaecae* also found to be significantly more abundant in post-stroke, rotenone-induced Parkinson Disease(12), and non-alcoholic fatty liver disease(127). Moreover, an increase of *Peptostreptococcaeca* is associated with metabolic disease after 30 days of high fat diet(128). Inflammation and low fiber diet related family *Erysipelotrichaceae* was more abundant in APP/PS1 mice of Alzheimer's diseases and PD-1 knockout mice with high chance of sepsis compare to C57BL/6 mice(129). Its abundance level were found to enrich in the lumen of colorectal cancer patients as compared to healthy controls(130). Previously in our lab, the abundance of *Enterococcus* and *Erysipelotrichaceae* were negatively correlated with cholic acid and octadecanedioic acid while positively associated with phosphatidylethanolamines and stearic acid in morphine-treated mice(60). It indicated that immune and metabolic pathways were both considered to play a part for exacerbating morphine analgesic tolerance.

In the chronic morphine-treated group, *Comamonadaceae*, *Coriobacteriaceae*, *Moraxellaceae*, and *Caulobacteraceae* were found significantly decreased. *Coriobacteriaceae* were found significantly decreased in patients with a higher pouchitis disease activity index(131). The pouchitis was also inversely correlated with *Moraxellaceae* indicated by erythrocyte sedimentation rate and fecal lactoferrin(132). The abundance of *Moraxellaceae* and *Lachnospiraceae* were found decreased in chronic alcohol-treated mice(133).

At genus level, the abundance of *Allobaculum* from the family of *Erysipelotrichaceae* were up-regulated. It has been reported that high cholesterol diet (HCD) induced changes in gut microbiota and inflammation. The *Allobaculum*, which was found increased after HCD, exhibited a negative correlation with colonic expression of anti-inflammatory genes such as Foxp3, IL-10, occludin and ZO-1(134). The *Dehalobacterium* were also found increased after high fat diet. It is correlated with intestinal inflammation and barrier disruption in mice with non-alcoholic fatty liver disease(135). The abundance of *Oscillospira* were found higher in old SPF mice. This age-related gut dysbiosis associated with intestinal permeability, systemic inflammation and macrophage dysfunction(136). However, *[Ruminococcus], Adlercreutzia, Acinetobacter, Brevudimonas, Delftia,* and *Turicibacter* were found decreased after morphine treatment. In the Multiple Sclerosis patients, *Adlercreutzia* with *Prevotella* were found decreased, indicating their contribution to the inflammatory processes in neurological disorders(137). However, Gram-negative bacteria *Acinetobacter* were found translocated into mesenteric lymph nodes, spleen and livers, involving in the neuroinflammation in rat depression model(138). *Turicibacter*, which abundance is decreased in die-induced obesity, associated with increased intestinal inflammation and alterations of the Wnt pathway towards tumorigenesis(139). Taken together, although the specific roles of these bacteria in morphine tolerance model have not been delineated, the growing literature implies that dysbiosis might have an influence on analgesic tolerance through modulating gut homeostasis including metabolism, immune activation and gut barrier disruption.

The dramatic depletion of *Bifidobacterium* and *Lactobacillus*, two essential and common inhabitants of the human intestine, were of particular interest because of their

roles in maintaining gut homeostasis and gut epithelial integrity(140). *Bifidobacterium* and *Lactobacillus* with anti-inflammatory and antioxidative properties are also shown to be significantly decreased in aging and in Alzheimer's patients with concomitant increase in intestinal permeability and inflammation(141). Together our studies imply the reduction in these two bacterial communities plays a crucial role in morphine tolerance. The enrichment of *Bifidobacteria* and *Lactobacteriaceae* by VSL#3 administration are resistant against pathogens and preserve the gut homeostasis. VSL#3 supplementation suppressed the abundance of these pathogens including *Peptostreptococcaceae*, *Erysipelotrichaceae*, *Prevotellaceae*, *Dehalobacteriaceae, Allobaculum, Prevotella, Dehalobacterium*. However, it could not inhibit the decreased of *Moracellaceae* and *Acinetobacter.* After administration of VSL#3 probiotics, *Lactobacillaceae* and *Bifidobacterium* were restored in the mice ileum. There are studies implying *Lactobacillaceae* were negatively associated with neuroinflammation in cirrhosis in mice(142). *Bifidobacterium longum NCC3001* attenuated anxiety-like behavior in mice with infectious colitis(44). In conclusion, microbiota as the "forgotten organ" is a key factor in maintaining gut and neuronal homeostasis. The imbalance of bacteria in the gut due to chronic morphine treatment contributes to analgesic tolerance.

In the pan-antibiotics treated mice, we observed less analgesic tolerance after chronic morphine treatment. Though a broad-spectrum antibiotic approach reduced the majority of bacterial species, there will still bacterial left in the gut that was not detectable by gel electrophoresis. Furthermore, although Pimaricin in pan-antibiotics combination was anti-fungi, the other bacteriophages and eukaryotic virus, which were

not directly targeted by bactericidal antibiotics, cannot be ignored. Thus, we used both broad-antibiotics treatment model and germ-free mice model.

Depletion of the gut microbiome with broad spectrum antibiotics and/or absence of the gut microbiome as in GF mice have revealed the critical role of the gut microbiome in the behavior, stress- and pain-modulation systems, and central neurotransmitter systems(20)(143)(144). The GF mice exhibited reduced anxiety-like behavior, impaired short-term recognition and working memory, increased stereotyped and repetitive behavior(145). GF mice also alleviated neurological diseases such as EAE and Alzheimer's disease(146)(147). The gut microbiome, as a potential contributor to morphine analgesic tolerance, was previously implicated in studies by Kang et al. wherein they demonstrated that pan-antibiotics attenuated morphine tolerance tested by tail immersion, acetic acid stretch and tail flick assays. Notably, the vancomycin along, which targets gram-positive bacteria, is sufficient to inhibit morphine tolerance generation. However, the combination of streptomycin, neomycin, vancomycin and metronidazole have the best effects(69). It indicates that gram-positive bacteria are pivotal for morphine-induced analgesic tolerance. Moreover, although pan-antibiotics could not change morphine concentration in the brain, it reversed morphine-induced reduction in electrical excitability of DRG neurons (69). In their following studies, they demonstrated that gut-derived mediators produced by morphine-induced dysbiosis were responsible for the development of morphine tolerance by regulating the DRG neuron through tetrodotoxin-resistant (TTX-R) Na+ and TRPV1 channels(148). Both our studies and their research demonstrated that morphine-induced dysbiosis provides a detrimental peripheral environment to exert its influence on CNS by producing

bacterial products, inducing proinflammatory cytokines, activating TLR2/4 and sensitizing of TTX-R Na+ channel.

In another study, the mice spend significantly more time in the cocaine-paired chamber after repeated cocaine injection, indicating cocaine abuse and dependence. However, antibiotics-treated mice did not show preference to cocaine-related chamber in conditioned place preference experiment. Moreover, mice receiving gut microbiota from morphine-treated mice exhibited no preference to cocaine. These studies imply that morphine-induced dysbiosis regulates rewarding and addiction, in addition to morphine tolerance(70).

Taken together, these studies provide strong support for the role of the gut microbiome in the development of morphine analgesic tolerance, addition and rewarding, and support targeting the microbiome as a druggable site for prolonging the analgesic efficacy of morphine for pain management.

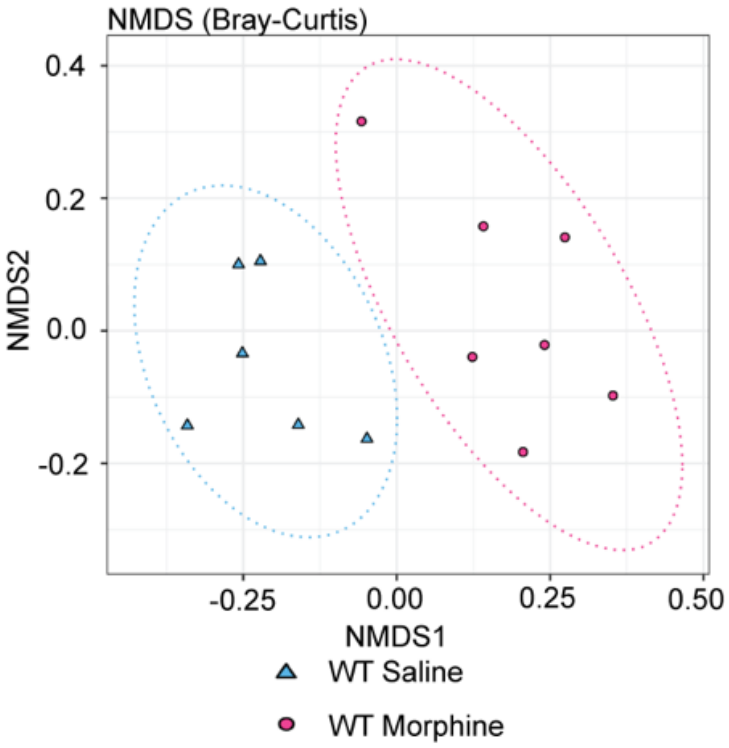


Figure 2.1 Multidimensional scaling analysis of gut microbiota to visualize the Bray-Curtis distance of WT morphine tolerant mice and their controls. Red circles depict samples from morphine-tolerant mice; blue triangles represent WT saline-treated mice. β-diversity was found to be significantly different between the WT morphine-tolerant and saline-treated groups ($p=0.00256$).

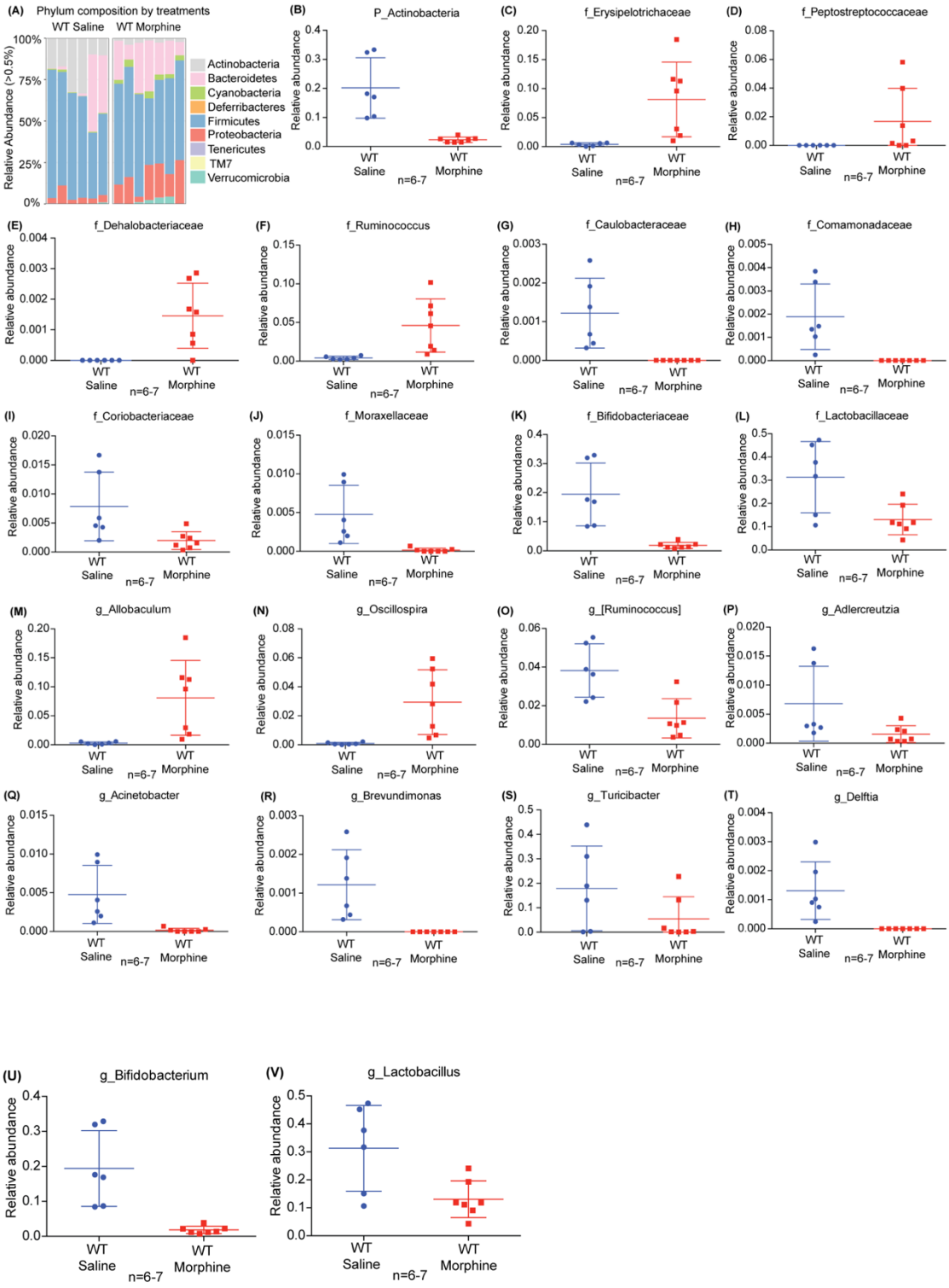


Figure 2.2 Microbiota alteration after 8 days of chronic morphine treatment. (A) Taxonomic distribution of WT morphine-tolerant mice and their controls at phylum level. Each column represents the fecal sample from one mouse. (B-V) Relative

abundance of each bacteria community in saline and chronic morphine treatment groups.

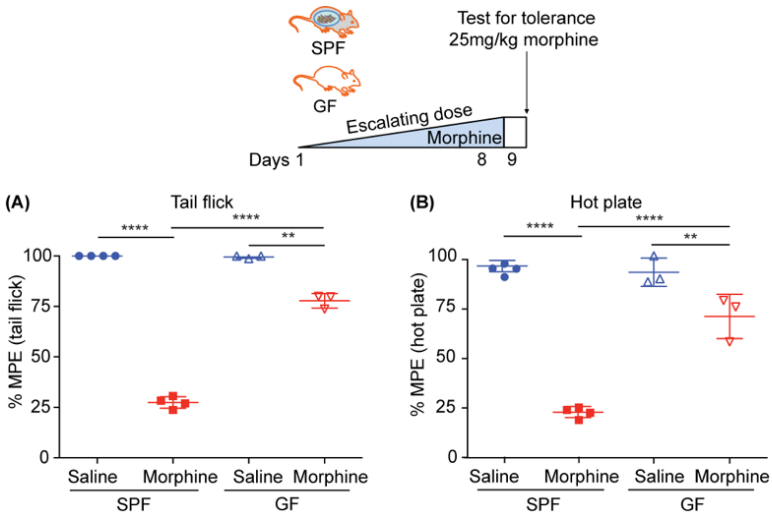


Figure 2.3 Morphine tolerance was attenuated in GF mice compared to SPF mice under the same dose of morphine treatment. (A, B) Antinociceptive tolerance was alleviated in GF mice. $F_{\text{tail flick}}$ (3,10) = 869.4 and $F_{\text{hot plate}}$ (3,10) = 110.9. Data were analyzed by one-way ANOVA followed by Bonferroni correction. **, $p<0.01$; ****, $p < 0.0001$.

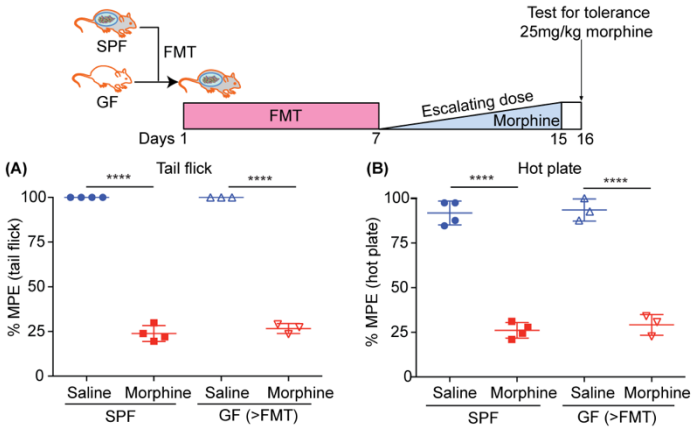


Figure 2.4 GF mice recapitulated morphine tolerance after FMT with naïve mouse microbiota. (A, B) The GF mice were gavaged with microbiota from naïve SPF mice for 7 days. Then the mice were administered escalating doses of morphine. $F_{\text{tail flick}}(3,10) = 882.8$ and $F_{\text{hot plate}}(3,10) = 147.8$. $n_{SPF}=4$, $n_{GF}=3$. Data were analyzed by one-way ANOVA followed by Bonferroni correction. ****, $p < 0.0001$.

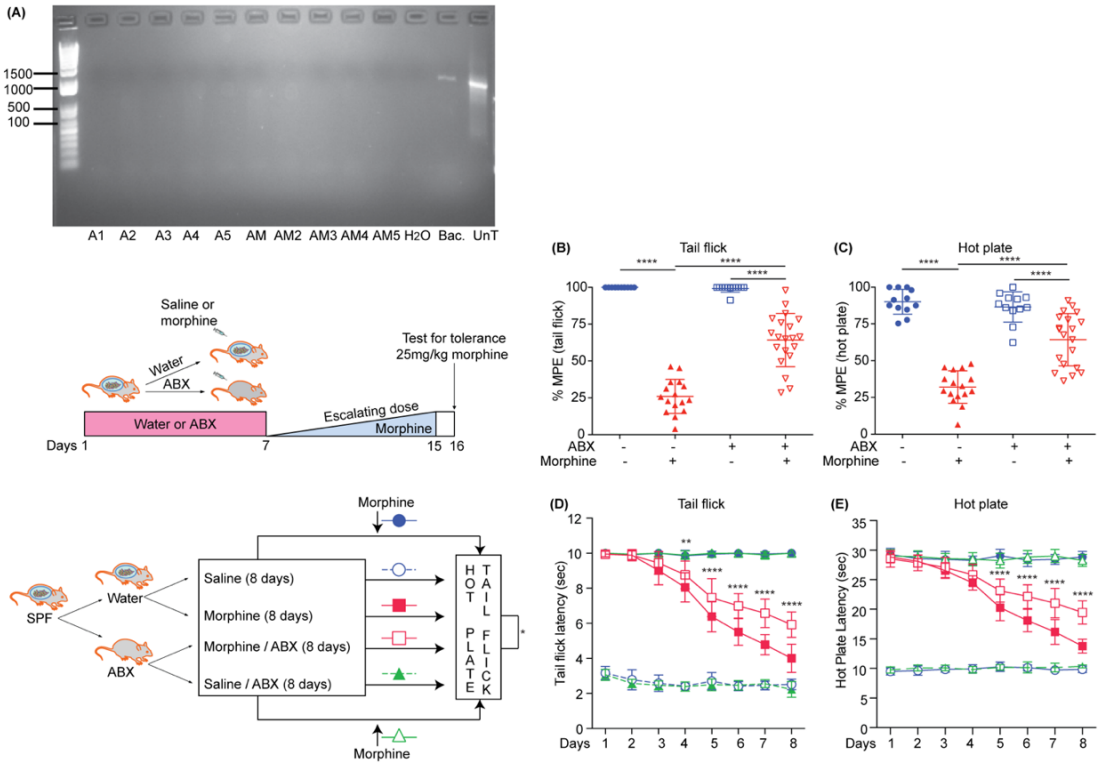


Figure 2.5 Analgesic tolerance was alleviated in ABX-treated mice. (A) Representative image of PCR product of bacterial 16S rRNA gene on electrophoresis gel. A: ABX + Saline-treated mice; AM: ABX + Morphine-treated mice; n=7 for each group. Bac., bacterial DNA; Untreated, ABX untreated mice as positive controls. (B, C) Mice exhibited attenuated analgesic tolerance after gut microbiota depletion. n=12-20. (D, E) Time course of ABX treatment on morphine tolerance. n=10-16, presented as Mean ± SD. $F_{Treatment\ x\ time}$ (35, 434) = 52.46 for tail flick. $F_{Treatment\ x\ time}$ (35, 434) = 60 for hot plate. Significance was assessed by two-way ANOVA followed by Tukey's multiple comparisons method. **, $p<0.01$, ****, $p<0.0001$.

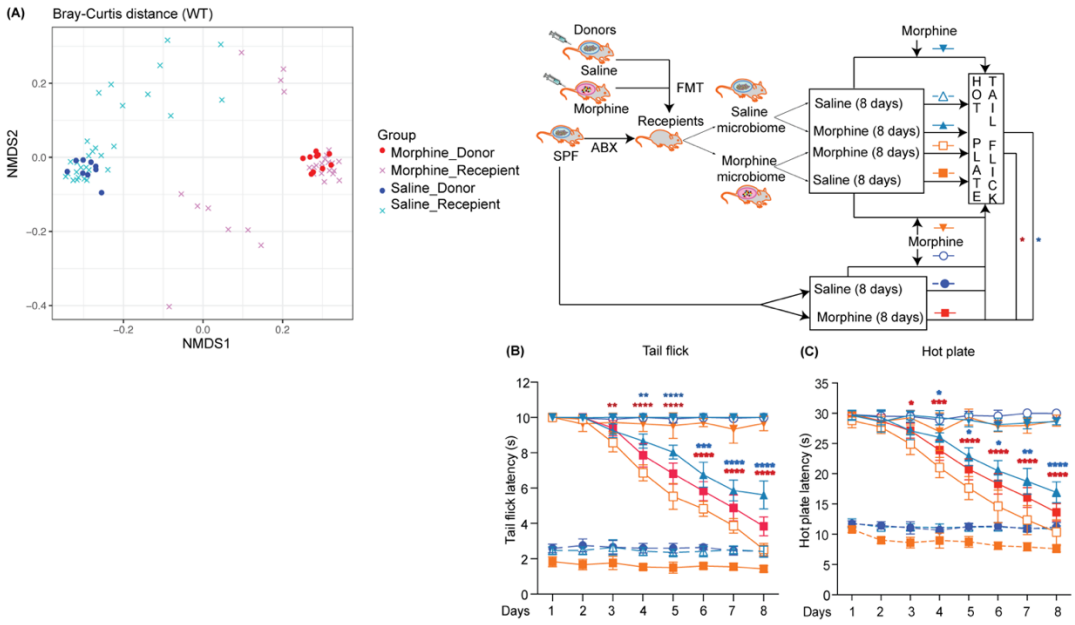


Figure 2.6 Morphine analgesic tolerance was regulated by gut microbiota manipulation. (A) Multidimensional scaling analysis plot of stool samples based on Bray-Curtis distance. The microbiome of recipient mice was similar to the donor mice after FMT (Saline_Recepient vs Saline_Donor, p=0.282; Morphine_Recepient vs Morphine_Donor, p=0.33612, Permutation ANOVA test). NMDS: non-metric multidimensional scaling. Time course of the effects of different microbiota on morphine tolerance. n=6-8. $F_{Treatment\ x\ time}$ (56, 343) = 73.15 for tail flick. $F_{Treatment\ x\ time}$ (56, 343) = 59.66 for hot plate. Two-way ANOVA followed by Tukey's multiple comparison was used. *, p<0.05; **, p<0.01; ***, p< 0.001; ****, p<0.0001 Morphine vs Saline microbiome + Morphine; *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001 Morphine vs Morphine microbiome + Morphine. Each dot represents one mouse. Mean ± SD. MPE: maximum possible effect.

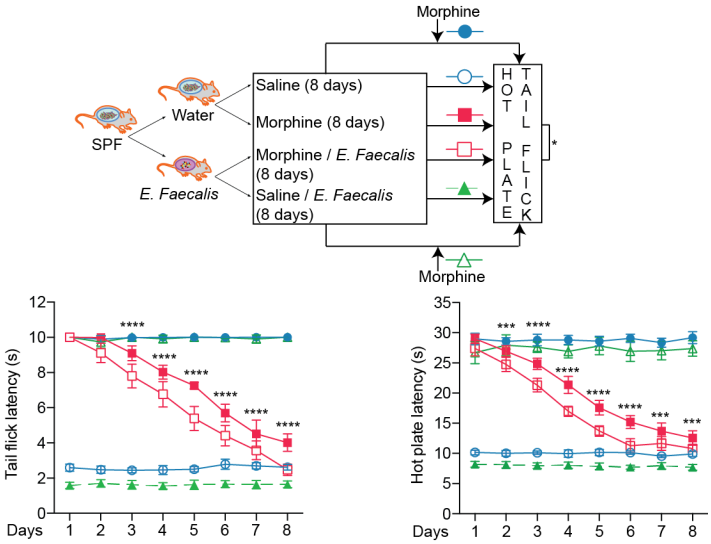


Figure 2.7 *Enterococcus faecalis* infection accelerated the generation of morphine analgesic tolerance. (A, B) Morphine analgesic effect was determined everyday by both tail flick and hot plate. n=6-10. Statistical analyses were carried out by two-way ANOVA followed by Tukey's multiple comparison method. $F_{\text{tail flick}}(35, 252) = 86.77$. $F_{\text{hot plate}}(35, 252) = 127.0$.

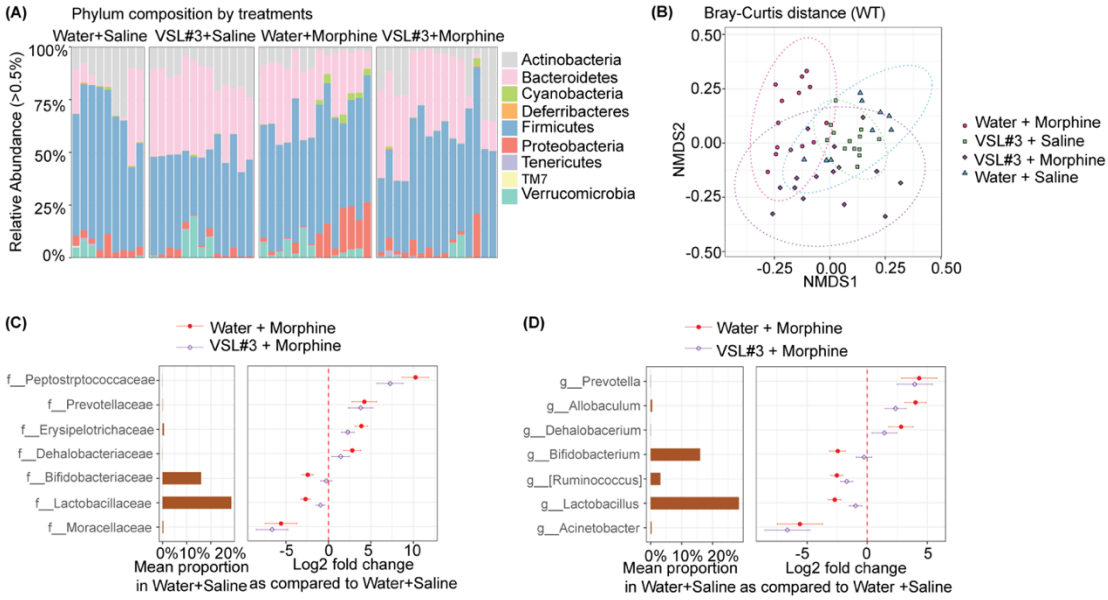


Figure 2.8 VSL#3 partially restored morphine-induced gut dysbiosis. (A) Multidimensional scaling was used to visualize the Bray-Curtis distance of different groups. n=9-15. Data were subjected to permutation ANOVA test along with Bonferroni correction. (B) Taxonomic distribution of different groups at phylum level. (C, D) Dot plots show changes in abundance of bacteria in Water+Morphine- and VSL#3+Morphine- treated mice at family and genus level using Water+Saline mean proportion as reference. n=9-15.

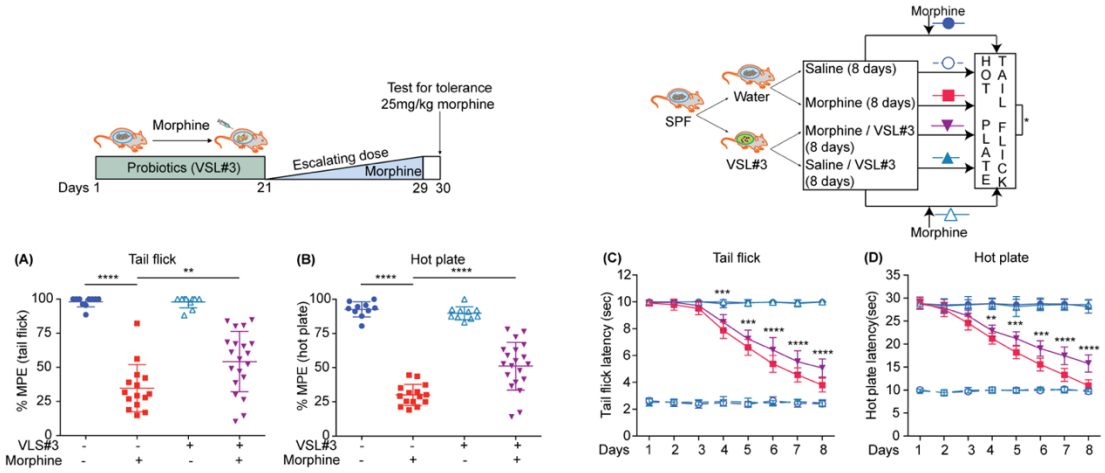


Figure 2.9 Probiotics VSL#3 pre-treatment attenuated morphine tolerance and prevented morphine-induced gut microbiota alteration. (A, B) Morphine analgesic effect was determined after escalating doses of morphine. n=10-20. $F_{\text{tail flick}}(3, 51) = 46.78$. $F_{\text{hot plate}}(3, 51) = 82.50$. One-way ANOVA followed by Bonferroni's correction was used to analyze data. (C, D) Time course of morphine tolerance was assessed by tail flick and hot plate tests. Mice were gavaged with probiotics and treated with constant doses of morphine. Data represent mean ± SD from 10-20 mice. Statistical analyses were carried out by two-way ANOVA followed by Tukey's multiple comparison method. $F_{\text{Treatment x time}}(35, 490) = 143.0$ for tail flick. $F_{\text{Treatment x time}}(35, 490) = 126.2$ for hot plate.

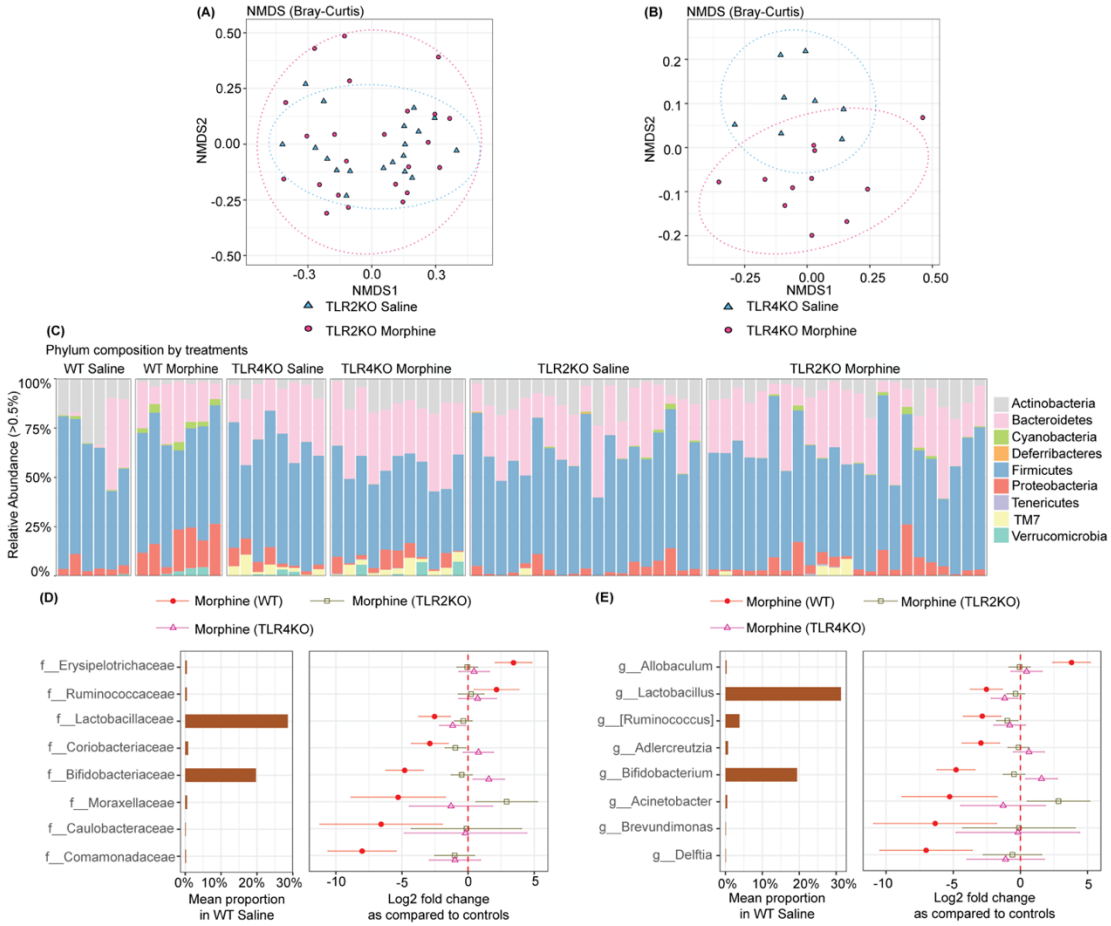


Figure 2.10 Morphine analgesic tolerance resulted in gut dysbiosis in WT mice, not in TLR2KO and TLR4KO mice. (A, B) Multidimensional scaling analysis of gut microbiota to visualize the Bray-Curtis distance of TLR2KO and TLR4KO morphine tolerant mice and their controls. Red circles depict samples from morphine-tolerant mice; blue triangles represent saline-treated mice. β-diversity was found to be significantly different between the WT morphine-tolerant and saline-treated groups ($p<0.001$). (C) Taxonomic distribution of TLR2KO and TLR4KO morphine-tolerant mice and their controls at phylum level. Each column represents a fecal sample from a treatment group. (D, E) dot plots show changes in abundance of bacteria with morphine treatment in WT, TLR2KO and TLR4KO at family and genus level using WT saline mean proportion as reference. Microbial taxa with significant difference in WT mice were selected at False Discovery Rate (FDR)<0.1 and average relative abundance of

WT control > 0.1%; n_{TLR2KO}=19-24; n_{TLR4KO}=8-11. NMDS: non-metric multidimensional scaling.

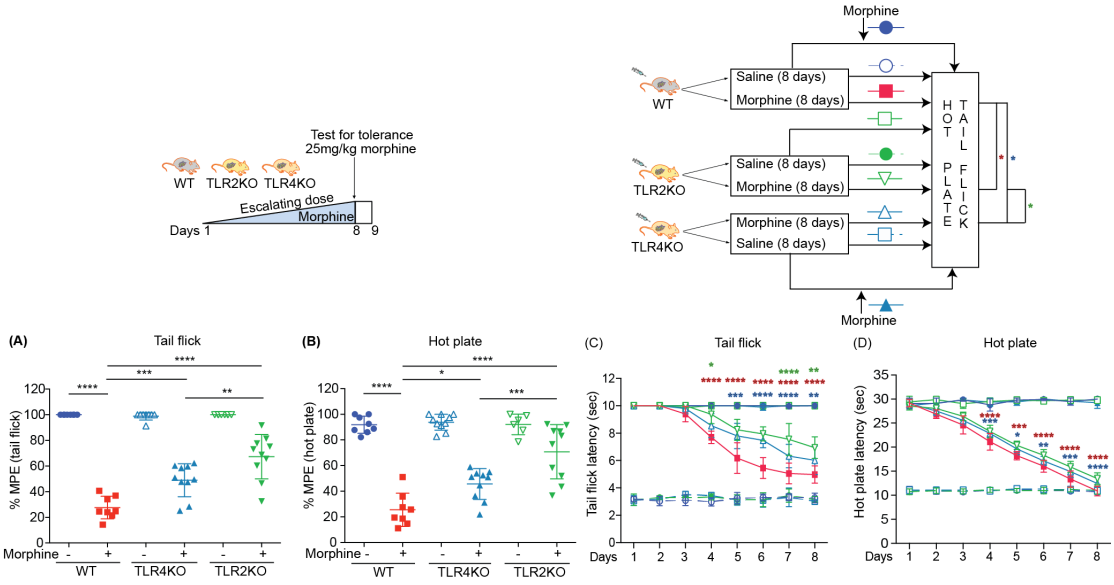


Figure 2.11 Both TLR2KO and TLR4KO exhibited alleviated analgesic tolerance. (A, B) Morphine analgesic tolerance was determined in WT, TLR2KO and TLR4KO mice. $F_{tail\ flick}$ (5, 46) = 75.71. $F_{hot\ plate}$ (5, 46) = 42.04. n=8-10. (C, D) WT, TLR2KO and TLR4KO mice were treated with constant doses of morphine twice daily. $F_{Treatment\ x\ time}$ (56, 329) = 23.09 for tail flick. $F_{Treatment\ x\ time}$ (56, 329) = 103.5 for hot plate. Data were analyzed by two-way ANOVA followed by Tukey's multiple comparison method. *WT Morphine compared to TLR2KO Morphine, *WT Morphine compared to TLR4KO Morphine, *TLR2KO Morphine compared to TLR4KO Morphine.

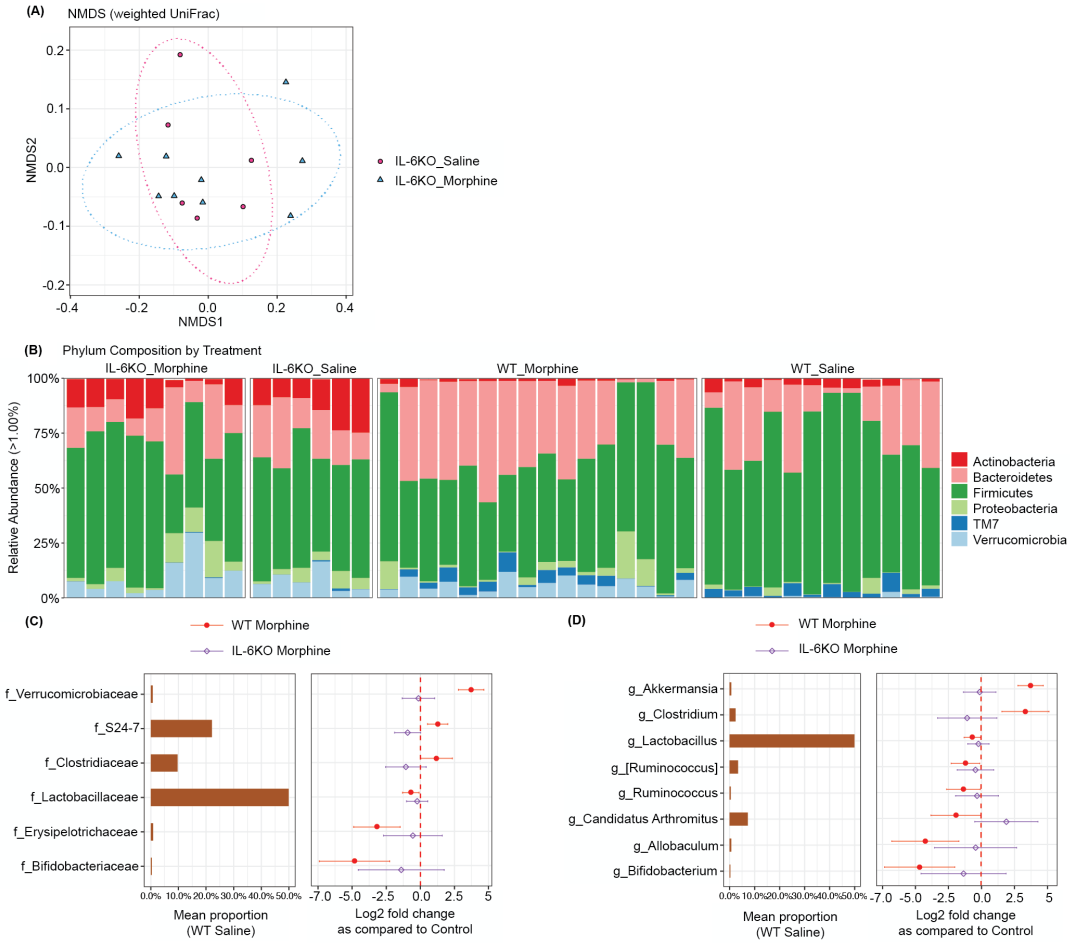


Figure 2.12 Chronic morphine-induced gut dysbiosis was abrogated in IL-6KO mice. (A) Multidimensional scaling analysis of gut microbiota to visualize the weighted Unifrac distance of saline- and morphine- treated IL-6KO mice. (B) Taxonomic distribution of IL-6KO and WT morphine-tolerant mice and their controls at phylum level. Each column represents one mouse sample from a treatment group. (C, D) dot plots show changes in abundance of bacteria with morphine treatment in WT and IL-6KO mice at family and genus level using WT saline mean proportion as reference. Microbial taxa with significant difference in WT mice were selected at False Discovery Rate (FDR)<0.1 and average relative abundance of WT control > 0.1%; $n_{IL\text{-}6KO}$=6-9; n_{WT}=12. NMDS: non-metric multidimensional scaling.

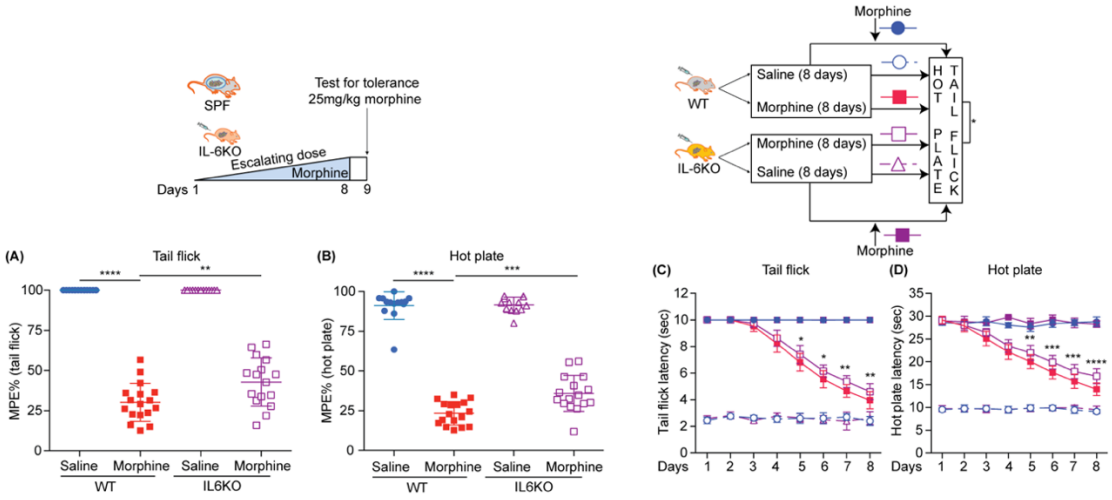


Figure 2.13 (A, B) WT and IL-6KO mice were treated with escalating morphine dosing for 8 days. n=13-17. $F_{\text{tail flick}}(3, 56) = 206.1$. $F_{\text{hot plate}}(3, 56) = 268.9$. Data were analyzed by one-way ANOVA followed by Bonferroni's multiple comparisons test. (C, D) WT and IL-6KO mice were treated with constant doses of morphine for 8 days. n=4-10. Data were analyzed by two-way ANOVA followed by Tukey's multiple comparison method. $F_{\text{Treatment x time}}(35, 321) = 83.33$ for tail flick. $F_{\text{Treatment x time}}(35, 321) = 79.3$ for hot plate. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$. Mean ± SD.

CHAPTER 3

THE MECHANISMS OF MORPHINE-INDUCED DYSBIOSIS CONTRIBUTING TO ANALGESIC TOLERANCE

INTRODUCTION

Morphine, as a narcotic pain reliever, is used to treat moderate to severe pain. However, morphine-induced analgesic tolerance often limits it usefulness in the long-term treatment of pain. The mechanisms underlying tolerance are not well understood. Studies on opioid analgesic tolerance have revealed several potential mechanisms: receptor desensitization and downregulation(101), upregulation of cAMP-protein kinase A (PKA) systems, release of glutamate, calcitonin-gene-related-peptide (CGRP), substance P (SP), noradrenaline, and acetylcholine to enhance synaptic transmission(149) and neuroimmune activation and neuroinflammation (75)(93)(150)(151)(152) (153) (154).

Recent studies show a strong association between inflammation and analgesic tolerance(75). It is postulated that chronic morphine treatment activates microglia cells and astrocytes to produce pro-inflammatory cytokines and chemokines IL-1β, TNF-α, IL-6, and CX3CL1(155). By acting in an autocrine/paracrine manner, the proinflammatory factors and their receptors are upregulated on immune cells, amplifying the mediators release and exacerbating morphine tolerance. Molecules and drugs are designed to determine the function of these inflammatory mediators and related signaling pathways in morphine tolerance. Resveratrol, Ibudilast and Minocycline are anti-inflammatory drugs have been shown to alleviate morphine tolerance(156)(157)(158). Thalidomide attenuates development of morphine dependence in mice by inhibiting PI3K/Akt and nitric oxide signaling

pathways(159). Glibenclamide suppresses NLPR3, thus inhibiting the activation of caspase1 to generate mature IL-1β(92). However, the precise mechanisms how inflammation regulates morphine tolerance have not been completely elucidated yet.

We have previously shown that morphine treatment compromises intestinal barrier function and induces bacterial translocation(59). Further studies demonstrated that morphine treatment induces a distinct shift of gut microbiota. Thus, we hypothesize that morphine-induced dysbiosis lead to gastrointestinal pathological changes and thus can have an impact on morphine analgesic tolerance. In this study, we aim to establish a link between gut dysbiosis and gut barrier compromise, bacterial translocation, and morphine analgesic tolerance.

Intestinal dysbiosis is a complication observed in a myriad of extra-intestinal disorders and diseases including neuropsychiatric disorders, tumor, metabolic disease, allergic disorder, and autoimmune diseases(116). Antibiotics are found to have neuroprotective properties against neurodegenerative and neuroinflammatory processes in Parkinson's Disease(18), Experimental Autoimmune Encephalomyelitis (EAE)(160), Multiple Sclerosis, and Transmissible Spongiform Encephalopathies(161). Furthermore, alcohol-induced neuroinflammation and small intestinal inflammation were abrogated following antibiotics administration(162). These studies highlight the fundamental role of gut microbiota in disease condition. However, it is still unknown whether morphine-induced dysbiosis contribute to morphine-induced neuroinflammation and thus analgesic tolerance. The current study therefore focuses on the role of morphine-induced dysbiosis on neuroinflammation as a mechanism contributing to analgesic tolerance.

Previously, Meng et al. demonstrated that morphine-induced bacterial translocation was alleviated in TLR2KO and TLR4KO mice. TLR2 and TLR4 modulate the tight junction organization in a MLCK-dependent manner. Therefore, less gut epithelial disruption was found in TLR2KO and TLR4KO mice(59). In addition, TLR2 and TLR4 are receptors for gram-positive and gram-negative bacteria, respectively. The pathogen associated molecular pattern (PAMP) such as LTA and LPS binds to TLR2 and TLR4 separately and initiates a cascade of events leading to significant immune response. Simultaneously, our lab found that morphine treatment enhances the TLR2 and TLR4 expression on microglia(163). Studies have indicated that damaged associated molecular patterns (DAMP) such as Heat Shock Protein (HSP)70, HSP90, High-mobility group box 1 (HMGB1) are generated during chronic morphine treatment. These PAMPs and DAMPs activate TLR2 and TLR4 on the microglia and astrocytes to produce cytokines such as TNF-α, and various interleukins such as IL-6, IL-1β, IL-8, IL-12 (92). Therefore, we hypothesize that gut dysbiosis and following bacterial translocation and inflammation contribute to analgesic tolerance through activation of TLR2 and TLR4.

To further prove the cross-correlation association between morphine-induced dysbiosis and morphine-associated abnormalities, we plan to use Fecal Microbiota Transplantation (FMT). For instance, cirrhotic patients suffer from persistent cognitive impairment. In the mouse model of cirrhosis, the mice undergoing FMT with microbiota from cirrhosis patients displayed greater neuroinflammation, microglial activation and GABAergic activation and lower synaptic plasticity compared to mice undergoing FMT with healthy controls(164). Furthermore, FMT, as a therapeutic

method, alleviates the symptom of neuropsychiatric disease and neurodegenerative disease. The FMT studies have been applied to ulcerative colitis, inflammatory bowel disease, Parkinson's disease, multiple sclerosis, myoclonus dystonia, chronic fatigue syndrome, and idiopathic thrombocytopenic purpura, which have shown favorable outcomes(165). Therefore, FMT of microbiota from chronic morphine-treated mice will allow us to gain insight into the mechanisms how gut microbiota contributes to morphine-induced gut pathogenesis and behavior changes. It might be an alternative therapeutic method to alleviate morphine-induced neuronal malfunction. Previously Banejee et al. studies have shown mice with FMT from morphine-treated mouse exhibited "morphine-like" diseased phenotype with damaged epithelial morphology, bacterial translocation and escalating IL-6 expression in liver(118). However, it is still unclear if there is a direct cause-consequence relationship between dysbiosis and morphine-induced neuroinflammation. Therefore, in this study, we plan to use the FMT approach to detect how morphine-induced dysbiosis exert its influence on TLR2/4 expression, bacterial translocation, gut integrity and peripheral and neuroinflammation.

The beneficial effects of probiotics are not only limited to gastrointestinal tract. In the gut, the underlying mechanisms of advantageous effects of probiotics include immunomodulatory effects, inhibition of pathogen colonization, production of bacteriocin-like inhibitory compounds against pathobiome, production of metabolites, lowering of pH and reinforcement of gut epithelial integrity(166). Therefore, probiotics contribute to central nervous system and behavior changes through gut-brain axis. For instance, the combination between *Bifidobacterium animalis* and *Lactobacillus plantarum* ameliorate neuroinflammation in multiple sclerosis by favoring Th2 and Treg differentiation via up-regulation of Foxp3 and GATA3(167). It has been shown

by our lab and other research groups that *Lactobacillus* was diminished in morphine-treated group(118)(70). Previously in chapter 2, we have demonstrated that morphine tolerance was alleviated after *Lactobacillus* was restored. However, it is not yet clear how *Lactobacillus* (VSL#3 probiotics) exerts its neuroprotective effect in chronic morphine model. Here we hypothesize that VSL#3 pretreatment suppresses morphine-induced neuroinflammation by alleviating the symptoms of bacterial translocation, gut epithelial integrity and intestinal inflammation.

In this study, we will use antibiotics-treated mouse model, germ-free (GF) mouse model, FMT model and probiotics treatment model to detect the role of morphine-induced dysbiosis in CNS abnormalities, therefore contributing to morphine tolerance. We hypothesized that morphine-induced dysbiosis has a detrimental effect on central nervous system by disrupting peripheral gut epithelial barrier function leading to bacterial translocation and local inflammation. We propose probiotics as a promising approach in the manipulation of the intestinal microbiota with tremendous potential applications in the morphine-induced behavior changes.

MATERIALS AND METHODS

Intestinal permeability and bacterial translocation:

To access *in vivo* intestinal permeability, 600 mg/kg of FITC-dextran (wt 4000; Sigma-Aldrich) was orally gavaged into mice 4 hour prior to blood collection. After sacrifice, serum FITC-dextran fluorescence intensity was measured by SpectraMax® i3x. MLN and liver tissue were collected, homogenized and cultured on BD™ Trypticase™ Soy Agar plates with 5% Sheep Blood (TSA II™) to determine bacterial translocation.

Real-Time PCR:

Total RNA from spinal cord and ileum was extracted using TRIzol (Invitrogen). cDNA was synthesized using the M-MLV Reverse Transcription kit (Promega) following manufacturer's protocol. Primers for IL-6, IL-1β, TNF-α, and GAPDH were purchased from Invitrogen. Quantitative real-time polymerase chain reaction was performed using LightCycler® 480 SYBR Green I Master (Roche). GAPDH was used to normalize values. The results were analyzed by the relative quantity (ΔΔCt) method. Primer sequences: GAPDH: 5'-ACGGCAAATTCAACGGCACAGTCA-3', 5'-TGGGGGCATCGGCAGAAGG-3'; IL-6: 5'-TGGCTAAGGACCAAGACCATCCAA-3', 5'-AACGCACTAGGTTTGCCGAGTAGA-3'; TNF-α: 5'-CCTCCCTCTCATCAGTTCTATGG-3', 5'-CGTGGGCTACAGGCTTGTC-3'; IL-1β: 5'-GGCAGGCAGTATCACTCATT-3', IL-1β: 5'-AAGGTGCTCATGTCCTCATC-3'.

PCR:

Bacterial DNA was isolated from mouse tissues as previously described. The 16S ribosomal RNA (rRNA) gene was amplified by 5'-TTGGAGAGTTTGATCCTGGCTC-3', and 5'-ACGTCATCCCCACCTTCCTC-3'. Gram-positive and gram-negative bacterial DNA was amplified by NF with N6R, and NF with NR and P2F, respectively. NF: 5'-GGCGGCAKGCCTAAYACATGCAAGT-3', NR: 5'-GACGACAGCCATGCASCACCTGT-3', P2F: 5'-GCGRCTCTCTGGTCTGTA-3', N6R: 5'-GGTGCCTTCGGGAAC-3'. Amplified DNA was visualized using 1% agarose electrophoresis gel(168).

Intestinal cell isolation and FACS:

Intestinal cell isolation was performed as previously described(169). Single cell suspensions were incubated with a mixture of antibodies: Pan-Keratin (C11) Mouse mAb (Alexa Fluor® 488 Conjugate, cell signaling), redFluor™ 710 Anti-Mouse CD45 (30-F11, Tonbo bioscience), Ghost Dye™ Red 780 (Tonbo bioscience), and either BV421 Rat Anti-Mouse CD282 (TLR2, BD bioscience) or BV421 Rat Anti-Mouse CD284 (TLR4, BD bioscience). The data were collected and analyzed by cytoExpert software with cytoFlex S (Beckman Coulter). The figures were made by Kaluza.

ELISA:

The concentrations of IL-6, IL-1β and TNF-α in brain, liver and MLN were quantified using enzyme-linked immunosorbent assay kits (Fisher Scientific).

Histology:

Sections of formalin-fixed paraffin-embedded gut tissues were subjected to H&E staining for the evaluation of morphine-induced morphological disruption of gut epithelial integrity as previously described(59).

Statistical analysis:

Experimental data were analyzed using Prism 6 (GraphPad Software, Inc.). Parametric data were compared using Student's t-test. For multiple-group comparison, data were analyzed by ANOVA one-way analysis, followed by Bonferroni correction or two-way analysis, followed by Tukey's multiple comparison method.

RESULTS

1. Gut microbiota was responsible for morphine-induced gut pathology.

After SPF mice were injected with 15mg/kg morphine twice daily for 8 days, ileum samples were dissected and examined for histopathological changes. In SPF morphine-tolerant mice, we observed impaired epithelia and increased inflammatory infiltrates in small intestinal villi (Fig. 3.1 A, B). In contrast, in morphine-treated GF and ABX mice, no morphological damage was observed (Fig. 3.1 C, D, E, F). Morphine-induced intestinal disruption was also seen in GF mice that received naïve microbiota from SPF mice (Fig. 3.1 G, H). In the GF or ABX mice undergoing FMT from saline- or morphine-tolerant mice, we observed that FMT from morphine-tolerant mice alone was sufficient to induce histopathological change in the gut without requiring direct exposure to morphine (Fig. 3.1 I, J, K, L).

2. Gut microbiota contributed to gut leakiness in morphine-tolerant mice.

To evaluate the disruption in gut permeability, SPF and ABX mice with or without FMT were gavaged with fluorescein isothiocynate (FITC)-dextran. Significant increase in gut permeability was observed in SPF morphine-tolerant mice (Fig. 3.2 A). Morphine's effect on gut permeability of SPF mice was abolished in ABX-treated mice (Fig. 3.2 A). In ABX mice that underwent FMT, a significant increase in FITC-dextran in the serum was observed in the mice receiving gut microbiota from morphine-tolerant mice (Fig. 3.2 B), but not in those receiving gut microbiota from saline-treated mice.

3. Gut microbiota attributed to bacterial translocation in morphine tolerant mice.

We next evaluated gut bacterial translocation to the liver and mesenteric lymph nodes (MLN) in the mice. Significant bacterial translocation was observed in SPF morphine-

tolerant mice (Fig. 3.3 A, B). As expected, morphine treatment failed to induce bacterial translocation in either GF or ABX mice (Fig. 3.3 A, B). However, when GF mice were reconstituted with the microbiota of naïve SPF mice, and then treated with morphine, bacterial translocation was observed (Fig. 3.3 C, D). In addition, when GF mice or ABX mice underwent FMT, the mice receiving morphine-tolerant microbiota showed significantly greater bacterial translocation than the mice receiving microbiota of saline-treated animals (Fig. 3.3 E, F). These studies suggest that morphine-induced gut integrity disruption and subsequent bacterial translocation is mediated by alterations in the gut microbiota. These results provide further evidence for the crucial role of morphine-induced dysbiosis in analgesic tolerance.

4. Gut microbiota was fundamental to increase TLR2 and TLR4 expression in morphine tolerant mice.

To further explore the characteristics of the translocated bacteria in the liver and MLN, we analyzed DNA isolated from the liver by gel-based PCR using the primers specific for gram-positive and gram-negative bacteria. We found both gram-positive and gram-negative bacteria translocating to the liver in the morphine-tolerant animals (Fig. 3.4 A). TLR2 and TLR4 are the major receptors that mediate the host's response to gram-positive and gram-negative bacteria, respectively. To determine the roles of TLR2 and TLR4 in morphine analgesic tolerance, we evaluated expression of these receptors on gut epithelial and immune cells. In morphine-tolerant animals, we found a significant increase in TLR2 and TLR4 expression in both gut epithelial cells (Fig. 3.4 B, C) and intraepithelial immune cells (Fig. 3.4 D, E). Furthermore, the expression of TLR2 and TLR4 was also significantly increased in circulating immune cells isolated from the blood of morphine-tolerant animals (Fig. 3.4 F, G). The expression levels of TLR2 and

TLR4 were not up-regulated by morphine treatment in ABX mice (Fig. 3.4 A, B, C and D). Surprisingly, in circulating immune cells, morphine increased TLR4 expression in GF mice (Fig. 3.4 G). However, in ABX mice, TLR4 expression on circulating immune cells was not altered by morphine (Fig. 3.4 G).

After reconstituting the GF mice with the naïve microbiome of SPF mice, morphine treatment induced tolerance in these mice, and elevated TLR2 and TLR4 expression on gut epithelial and immune cells (Fig. 3.4 H, I, J, K, L, M). Moreover, transplantation of microbiota from morphine-tolerant mice alone was sufficient to enhance TLR2 and TLR4 protein expression in GF and ABX mice (Fig. 3.4 N, O, P, Q, R, S). These data suggest that TLR2 and TLR4 activation, as a consequence of dysbiosis and bacterial translocation, and the resulting induction of proinflammatory cytokines may be mediators of morphine analgesic tolerance.

5. Neuroinflammation and small intestinal local inflammation required gut microbiota in morphine tolerant mice.

Accumulating evidence implicates inflammation as a contributing factor in morphine-induced analgesic tolerance. To further investigate the role of inflammatory cytokines in this process, animals were subjected to repeated morphine injections. In morphine-tolerant animals, the protein levels of pro-inflammatory cytokines IL-6, IL-1β and TNF-α were measured in the liver, MLN, and brain by enzyme-linked immunosorbent assay (ELISA). Additionally, messenger RNA levels of these cytokines were also determined in the spinal cords and intestines. Morphine-tolerant animals displayed significantly elevated levels of these cytokines in all tissues examined. However, morphine administration to GF and ABX mice showed less inflammatory response (Fig.

3.5 A, B, C, D, E, F, Table 1). GF mice receiving naïve SPF microbiota developed analgesic tolerance and displayed higher expressions of these cytokines following morphine treatment (Fig. 3.5 G, H, I, J, K, L, Table 2). FMT of the gut microbiota from morphine-tolerant mice into GF and ABX recipients resulted in increased expression of these cytokines. (Fig. 3.5 M, N, O, P, Q, R, Table 3). Together, these studies demonstrate that alteration in microbial compositions disrupted gut integrity, facilitated bacterial translocation, regulated TLR expressions and activation, and exacerbated inflammation, all contributing to morphine tolerance.

6. TLR2KO and TLR4KO mice exhibited less gut leakage, fewer bacterial translocation and diminished inflammation after chronic morphine treatment.

The TLR2KO and TLR4KO mice, as previously described, exhibited attenuated morphine tolerance after 15mg/kg morphine twice daily administration. Compared to WT morphine-treated mice, both TLR2KO and TLR4KO mice displayed less impaired gut integrity (Fig. 3.6 A) and lower bacterial translocation (Fig. 3.6 B, C) after morphine treatment. Repeated morphine injections into TLR2KO and TLR4KO mice induced less inflammation than equivalent injections into WT mice (Fig. 3.6 D, E, F, G, H, I, Table 4).

7. IL-6KO mice exhibited less gut morphological changes and bacterial translocation after chronic morphine treatment.

The IL-6KO mice, which have attenuated morphine tolerance as previously described, have alleviated gut histological damage (Fig. 3.7 A) and bacterial translocation (Fig. 3.7 B, C) compared to WT control.

8. VSL#3 probiotics attenuated chronic morphine-induced morphological damage and following gut leakage and bacterial translocation.

As previously described, the VSL#3-treated mice exhibited less tolerance than sham mice after chronic morphine injection. The VLS#3 probiotics restored morphine-induced dysbiosis, especially reinstalling the abundance of *Lactobacillaceae* and *Bifidobacteriaceae*. Comparing with the morphine-treated mice, the gut histology showed less immune cell infiltration and gut epithelial damage in VSL#3 + morphine-treated mice (Fig. 3.8 A). Morphine-induced gut permeability (Fig. 3.8 B) and systemic bacterial translocation into liver and MLN (Fig. 3.8 C, D) were rescued using probiotics treatment.

9. VSL#3 probiotics attenuated chronic morphine induced TLR2 and TLR4 expression increase.

Furthermore, morphine tolerance-induced increases in TLR2 and TLR4 expression on the gut epithelia, gut and systemic immune cells (Fig. 3.9 A, B, C, D, E, F) which were also ameliorated by probiotics pre-treatment.

10. VSL#3 probiotics attenuated chronic morphine-induced intestinal and spinal cord inflammation.

The morphine-induced elevated proinflammatory cytokines IL-6 (Fig. 3.10 A, B), IL-1β (Fig. 3.10 C, D) and TNF-α (Fig. 3.10 E, F) in intestine and spinal cord, which were also ameliorated by probiotics pre-treatment. These data clearly support the use of probiotics as an adjunct therapy in patients using opioids for pain management.

DISCUSSION

In the present study, different mouse models were used to demonstrate that the gut microbiota plays an essential role in TLR2 and TLR4 expression and activation, gut epithelial integrity, bacterial translocation, inflammation, therefore contributing to morphine tolerance. We show that morphine-induced dysbiosis promotes gut permeability and bacterial translocation. The translocated bacteria and bacterial components circulate into system, activate systemic TLR2 and TLR4 on the immune cells and assist inflammation, therefore generating and maintaining morphine analgesic tolerance. To establish cause-effect relationship, both GF mice and an antibiotic bacterial depletion strategy were used. Both in GF and ABX treated mice morphine treatment did not result in significant bacterial translocation. Hence no inflammation was generated to facilitate morphine tolerance. However, when morphine-induced dysbiosis was restored by probiotics treatment, less bacterial translocation occurred, and less inflammation were detected in both intestine and spinal cord.

In the GF mice, we observed analgesic tolerance with less neuroinflammation. One explanation is that there are no bacteria translocated from gut lumen after chronic morphine treatment. Therefore, TLR2 and TLR4 are not activated and less pro-inflammatory cytokines are generated that contribute to neuroinflammation. Another explanation is that proinflammatory gene expression is attenuated in GF mice because of their abnormal microglial cells(170). Although GF mice have more microglial cells, they are defective in GF mice in response to bacterial or viral challenge, indicating that the microbiota is required to respond to pathogenic infection in both the periphery and the central nervous system. These microglia impairment could be restored by microbiota or SCFA(145)(170). Antibiotic-treated SPF mice also exhibit immature

microglia similar to GF mice(170). Although we have not detected microglial activation in morphine-treated GF and ABX mice, modulation of microglia function in GF mice and in ABX treated mice may explain the decrease neuroinflammation that is observed in GF and ABX-treated mice following chronic morphine challenge. In addition, Fagundes et al (2008) demonstrated diminished inflammatory hypernociception in GF mice after carrageenan challenge. GF mice also displayed reduced perception of pain after TNF-α and IL1-β inflammatory stimulation. These findings implicated an important role of commensal microbiota for the host in the perception of pain(144).

Furthermore, GF mice exhibited increased adult hippocampal neurogenesis(171). Chronic morphine treatment had negative impact on neuronal differentiation, neurite outgrowth and survival of adult hippocampal neural progenitor cells in conventionally raised mice(172). It implies that GF mice may have more hippocampal neurogenesis to compensate for the adverse effects of morphine on hippocampal neurons, therefore displaying less morphine tolerance. Moreover, GF mice were found to have decreased levels of N-methyl-D-aspartate (NMDA) receptors, specifically NR1 and NR2 subunits(173)(174), whose expression upregulation and activation have been suggested to promote morphine tolerance(175). In addition, one of the key regulators of morphine tolerance is BDNF, which was found to be less in the cortex and hippocampus of GF animals compared with control(174). Taken together, it has been demonstrated that the gut microbiota is essential for optimal brain functioning which are evident from GF mice studies. Thus, alteration of these factors besides inflammation have to be taken into consideration when interpreting data on delineating mechanisms underlying morphine-induced analgesic tolerance in GF mice.

It has been reported that GF and ABX animals have reduced expression of TLR1 and TLR2, but similar expression of TLR4 and TLR6 in the gut. The recolonization of gut microbiota into GF mice and ABX mice augment the expression of TLR1 and TLR2(176). In our chronic morphine treatment model, TLR2 and TLR4 expression on both gut epithelial cells and immune cells were increased. As a consequence, morphine-induced bacterial translocation was observed leading to activated systemic immune cells. However, these phenomena were not obvious in GF and ABX animals. The increased TLR2 and TLR4 expression and activation are recapitulated after GF mice were recolonized with microbiota from morphine-treated mice. It indicates that the activation and higher expression of TLR2 and TLR4 is due to morphine-induced dysbiosis. In addition, the GF mice have reduced IgA secretion, and fewer and smaller Peyer's patches(145). In the cirrhosis model, the GF mice had less IL-1β and TNFα in the serum compared to conventional cirrhotic mice(142). In our chronic morphine model, IL-6, IL-1β and TNFα were attenuated in both small intestine and spinal cord in GF mice. Reduced proinflammatory cytokines might explain decrease in morphine tolerance in these mice.

Our studies using FMT provided the most convincing evidence for the role of microbiota in gut-immune-CNS signaling. In our studies, we used both GF mouse model and ABX mouse model. In both FMT models, we demonstrated the microflora alteration was sufficient to initiate detrimental outcomes of chronic morphine treatment, thus contributing to morphine-induced analgesic tolerance. Furthermore, daily gavage of saline microbiome significantly alleviated the development of morphine tolerance. It implied that symbiotic bacteria can protect against morphine-induced dysbiosis, thus maintaining CNS health and proper behavior. In addition, we found that sustained lower

expression of epithelial TLR2 and TLR4 in axenic and antibiotic-treated mice subsequently recovered in saline-microbiota-recolonized mice, and pathogenically enhanced in morphine-microbiota-recolonized mice and saline-microbiota-recolonized mice with chronic morphine treatment, suggesting the microflora regulate host behavior.

The breakdown of gut homeostasis caused by gut dysbiosis, and the consequently increased intestinal permeability and bacterial translocation, was increasingly considered to be the ultimate source of the systemic immune activation and neurological disturbances. The “leaky gut” and enteric bacterial translocation were consistently observed in autoimmune diseases such as type 1 diabetes, inflammatory bowel disease, neurobehavioral changes in obesity, chronic fatigue syndrome, stress, Autism Spectrum Disorder, frailty, schizophrenia, Alzheimer’s disease and brain dysfunction after sepsis(177)(178). Dysbiosis-induced production and secretion of compounds such as LPS and LTA reduced the tightness of the intestinal barrier, facilitating the contact of the pathogenic bacteria with submucosal lymphoid tissue. As a result, systemic inflammatory reactions occurred, which initiated neuroinflammation and, ultimately pathophysiology of these diseases. Thus, targeting intestinal integrity could provide new therapeutic possibilities of neuroinflammation and morphine-induced analgesic tolerance.

Recent studies have shown the benefits of probiotics as a means to restore and maintain health in diseased states such as allergic disease, diarrhea, irritable bowel disease, Alzheimer’s disease, anxiety, stress and depression(179)(180)(181)(182)(183). A recent report showed an increase in OPRM1 expression in human HT-29 epithelial cells following treatment with *Lactobacillus* (especially *L. acidophilus NCFM*)(184). These

studies further suggested that commensal bacteria can control the transmission of nociceptive information of the intestinal nervous system through OPRM1. It is likely that loss of these commensals, along with reduced expression of OPRM1 on epithelial cells, may contribute to reduced antinociceptive signals and heightened pain. In addition, VSL#3 probiotics have been shown to inhibit disease-associated cytokines IFN-γ, IL-17, and IL-6 while increasing anti-inflammatory cytokines TGF-β, IL-10 and IL-4 in EAE(167). Furthermore, VSL#3 probiotics were reported to ameliorate age-related deficits and reverse the effects of genes associated with aging(185). Furthermore, Zareie et al. (2006) also found that pretreatment of *Lactobacillus helveticus* and *Lactobacillus rhamnosus* prevented mucosal barrier dysfunction and bacterial translocation in chronic stress model, indicating probiotics could abrogate chronic psychological stress-induced intestinal abnormalities(183). Consistent with these studies, our studies found that decreases in *Lactobacillus* and *Bifidobacterium* play a crucial role in gut inflammation and are thus implicated in morphine tolerance. Our studies support the protective effect of probiotics on reinstating morphine effectiveness through the gut-brain axis in a morphine-tolerant model.

Previous literature showed that increased TLR2 and TLR4 mRNA and protein level on microglia in CNS after morphine exposure(163). It is noteworthy that TLR4 activation disrupted extracellular glutamate homeostasis between synapse owing to decreased glutamate uptake by astrocyte glutamate transporters, creating an environment of neuroexcitability(155). In addition, opioid-induced astrocyte and microglial activation leads to the release of many inflammatory cytokines not limited to IL-6, IL-1β, and TNFα, interleukins, interferons, monocyte chemotactic protein-1, CCL2, CCL3, CCL5, CXCL10, inducible nitric oxide synthase(79). These inflammatory molecules can

induce the production of a variety of neuroexcitatory substances such as nitric oxide, prostaglandin E2, and reactive oxygen species that modulate neuronal functioning(79). It has been previously shown that blockade of TLR4 attenuates the development of morphine tolerance by down-regulation of glial GLT-1 and GLAST glutamate transporters and proinflammatory cytokines production(186). TLR2 on microglial cells also contribute to morphine withdrawal. It was reported mice deficient in TLR2 inhibited morphine-induced microglia activation and dependence(187). However, the role of peripheral TLR2 and TLR4 function in morphine induced analgesic tolerance has been not reported. Previously in our lab, we demonstrated that the increased expressions of TLR2 and TLR4 on gut epithelia by morphine is involved in disrupting tight junctions, leading to leaky barrier and allowing the ingress of bacteria(59). In the present study, the expression of TLR2 and TLR4 were also found to be elevated in gut immune cells and systemic leukocytes after morphine administration. Moreover, morphine-induced bacterial alteration is sufficient to enhance TLR2 and TLR4 expression and activate TLR2 and TLR4 signaling pathway. The presence of bacteria in liver and MLN induced increase in systemic TLR2 and TLR4 expression and activation of these receptors lead to the production of proinflammatory cytokines IL-6, IL-1β and TNF-α.

Interestingly, in our previous studies and also in the current study showed that TLR2KO and TLR4KO mice were protected from microbial shift by morphine treatment. Attenuation of analgesic tolerance was detected in both TLR2KO and TLR4KO mice. Our lab has shown that lipid metabolites, especially bile acids, exhibited significant changes after morphine treatment. One of the major observations was a significantly higher level of cholesterol and its derivative coprostanol in morphine-treated WT mice.

However, the basal cholesterol levels in TLR2KO mice were significantly lower than their WT counterparts(118). On the other hand, concentrations of the host-derived primary and microbe-converted secondary bile acids were seen to diminish significantly in the feces of WT morphine-treated mice. However, the morphine-mediated changes were completely abolished in the TLR2KO animals(118). Therefore, we speculated that indigenous microflora regulates its host through modulating lipid metabolism, thus having an effect on morphine-induced behavior changes. However, how TLR2 modulation of lipid metabolism confers a protective role in morphine-induced tolerance needs further investigation.

In conclusion, the murine model of morphine analgesic tolerance showed an important link between the neuroinflammation and gut microbiota. Notably, the pan-antibiotic-treated and GF mice had ameliorated neuroinflammation, whereas microbial re-colonization with morphine-treated mice restored these functions. In our present study, we speculated that morphine-induced inflammation is a consequence of gut-host interaction. Changes in the composition of the gut microbiome, termed gut dysbiosis, leads to the breakdown of immune homeostasis in the intestinal immune system, which in turn induce distal abnormalities in the systemic immune and inflammatory pathways and thus playing a significant role in the development of morphine tolerance.

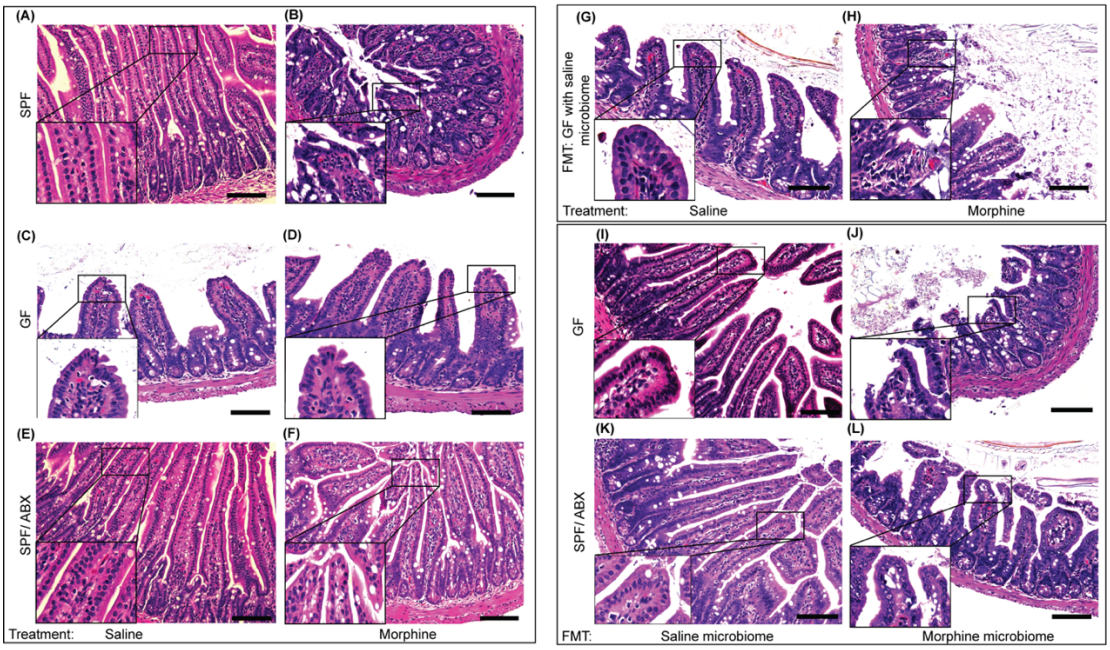


Figure 3.1 Morphine and morphine-induced dysbiosis cause gut epithelial damage. (A-F) SPF, GF and pan-antibiotics-pretreated SPF (SPF/ABX) mice were treated with saline or chronic morphine for 8 days. (G-H) GF mice were FMT with naïve microbiota, then treated with chronic morphine. (I-L) The GF or SPF/ABX mice were transplanted with gut microbiota from either saline or morphine-treated mice. Representative H&E stained section of mouse ileum. Scale bar: 100μm. WT and ABX mice: n_{SPF}=10, n_{GF}=3.

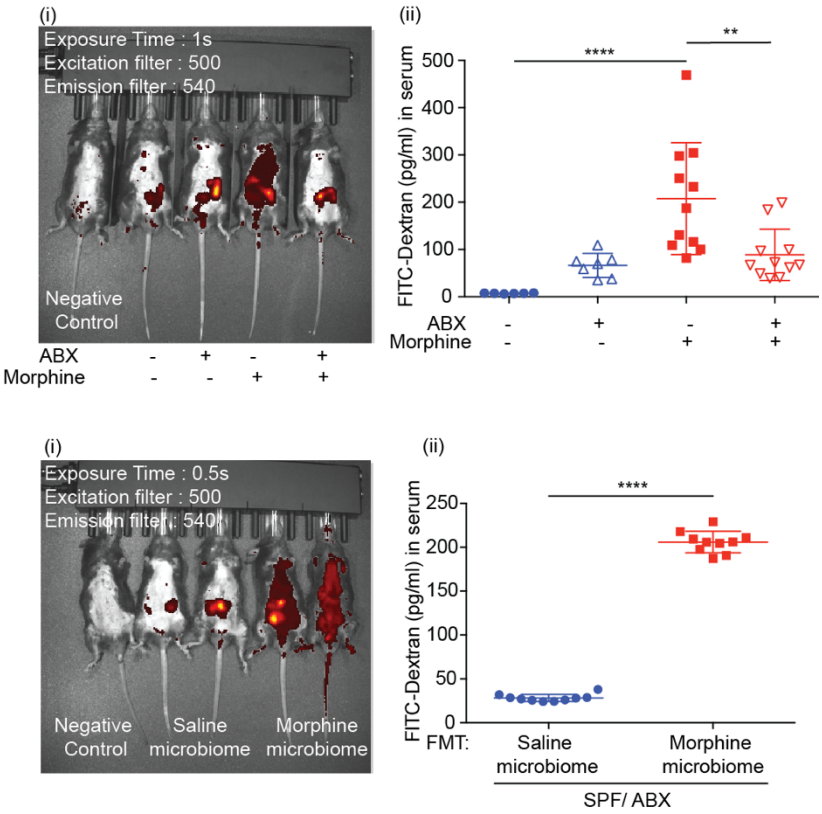


Figure 3.2 Chronic morphine treatment and morphine-induced microbiota alteration induce gut permeability. (A, B) Representative images and summary of fluorescein isothiocyanate (FITC)-dextran fluorescent signal distribution in mice. n=6-11.

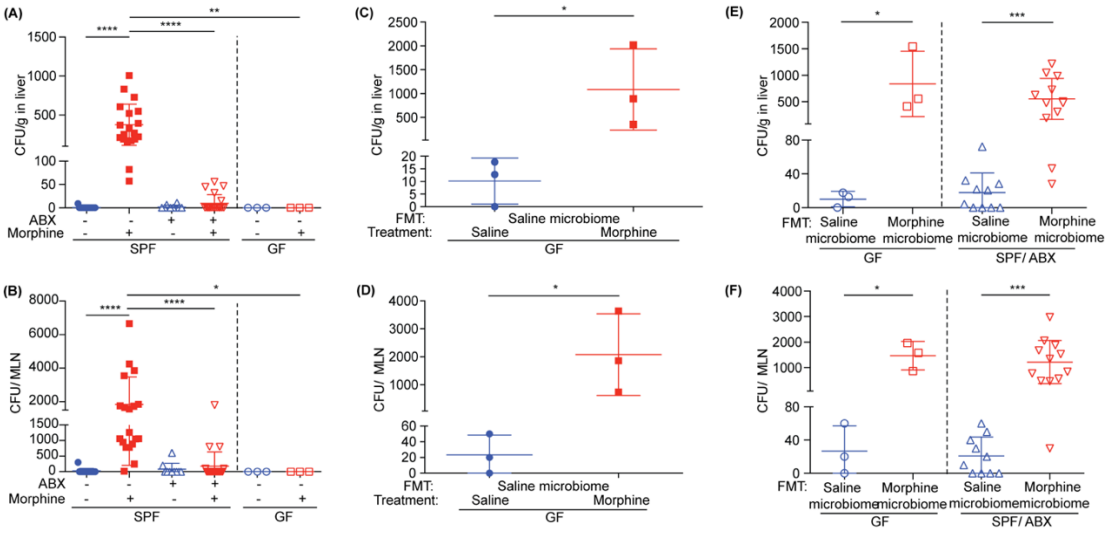
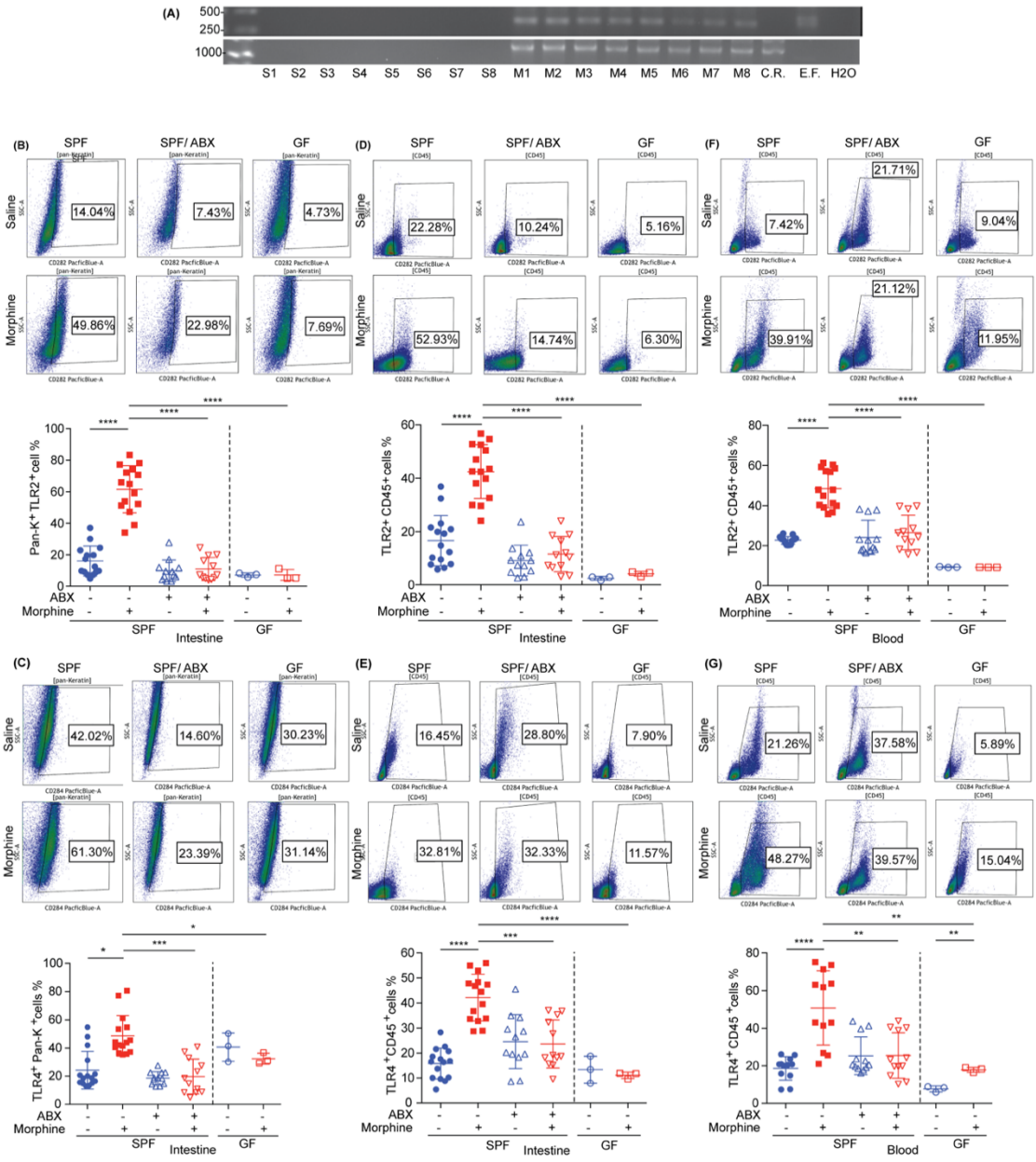
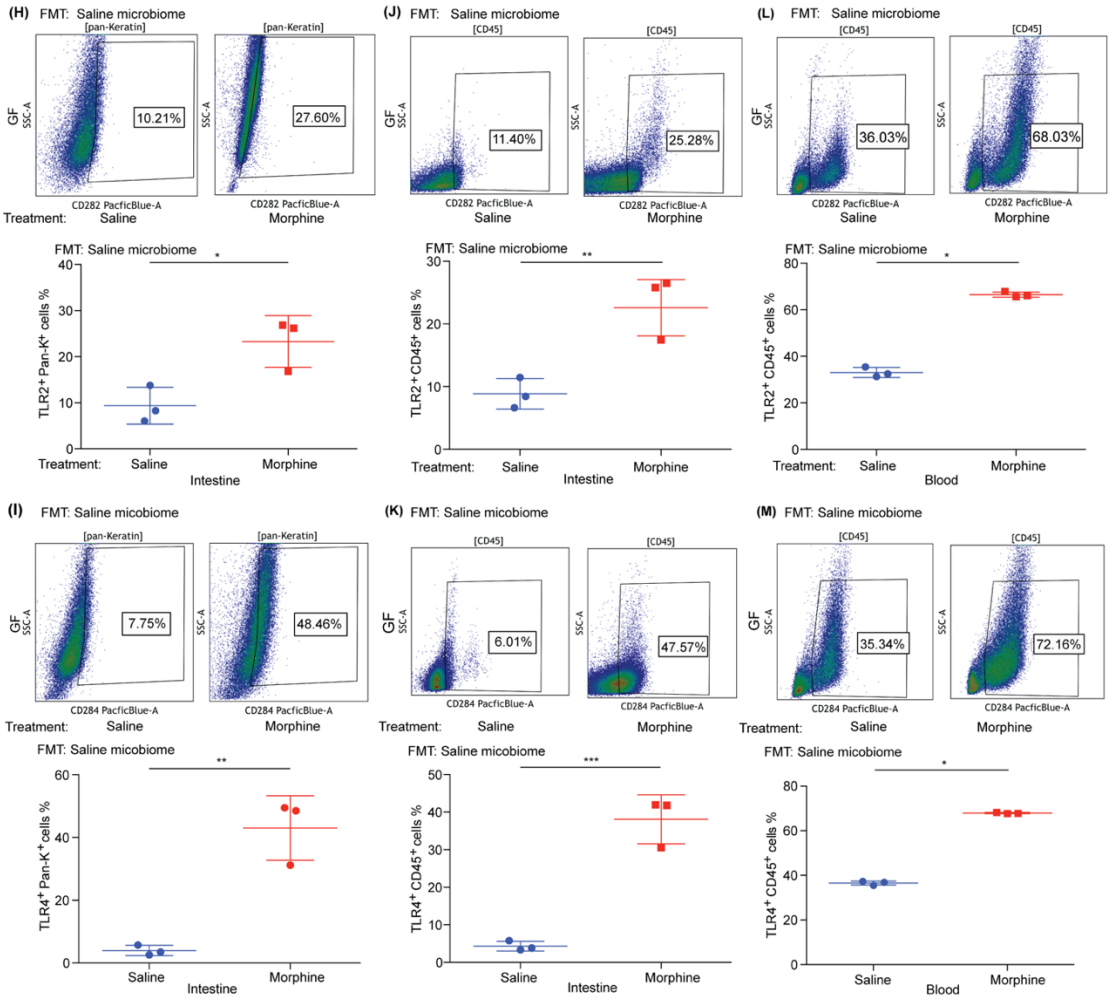


Figure 3.3 Morphine-induced dysbiosis is responsible for bacterial translocation. (A, B) Bacterial colony forming unit (CFU) in liver and MLN homogenates of GF and SPF mice. n_{SPF}=10-20; n_{GF}=3. Data were analyzed by one-way ANOVA followed by Bonferroni's multiple comparisons test. (C, D) CFU from GF mice reconstituted with normal gut microbiota and treated with either morphine or saline. n_{GF} = 3. (E, F) CFU from GF mice and ABX-treated SPF mice received gut microbiota either from saline or morphine tolerant- donors. n_{GF} = 3, $n_{SPF/ABX}$ = 10-12. Data were analyzed by student's t-test. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$, ****, $p<0.0001$. Mean ± SD.


(A)
500
250
1000
S1 S2 S3 S4 S5 S6 S7 S8 M1 M2 M3 M4 M5 M6 M7 M8 C.R. E.F. H2O
(B)
SPF
SPF/ ABX
GF
Saline
Morphine
14.04%
7.43%
4.73%
49.86%
22.98%
7.69%
Pan-K+ TLR2+ cell %
ABX
Morphine
SPF
Intestine
GF
(D)
22.28%
10.24%
5.16%
52.93%
14.74%
6.30%
TLR2+ CD45+ cells %
(F)
7.42%
21.71%
9.04%
39.91%
21.12%
11.95%
TLR2+ CD45+ cells %
Blood
(C)
42.02%
14.60%
30.23%
61.30%
23.39%
31.14%
TLR4+ Pan-K+ cells %
(E)
16.45%
28.80%
7.90%
32.81%
32.33%
11.57%
TLR4+ CD45+ cells %
(G)
21.26%
37.58%
5.89%
48.27%
39.57%
15.04%
TLR4+ CD45+ cells %


(H) FMT: Saline microbiome
[pan-Keratin]
GF SSC-A
10.21%
27.60%
CD282 PacficBlue-A
Treatment: Saline Morphine
FMT: Saline microbiome
TLR2+ Pan-K+ cells %
Treatment: Saline Morphine
Intestine
(J) FMT: Saline microbiome
[CD45]
11.40%
25.28%
TLR2+ CD45+ cells %
(L) FMT: Saline microbiome
36.03%
68.03%
Blood
(I) FMT: Saline micobiome
7.75%
48.46%
CD284 PacficBlue-A
TLR4+ Pan-K+ cells %
(K) FMT: Saline micobiome
6.01%
47.57%
TLR4+ CD45+ cells %
(M) FMT: Saline micobiome
35.34%
72.16%

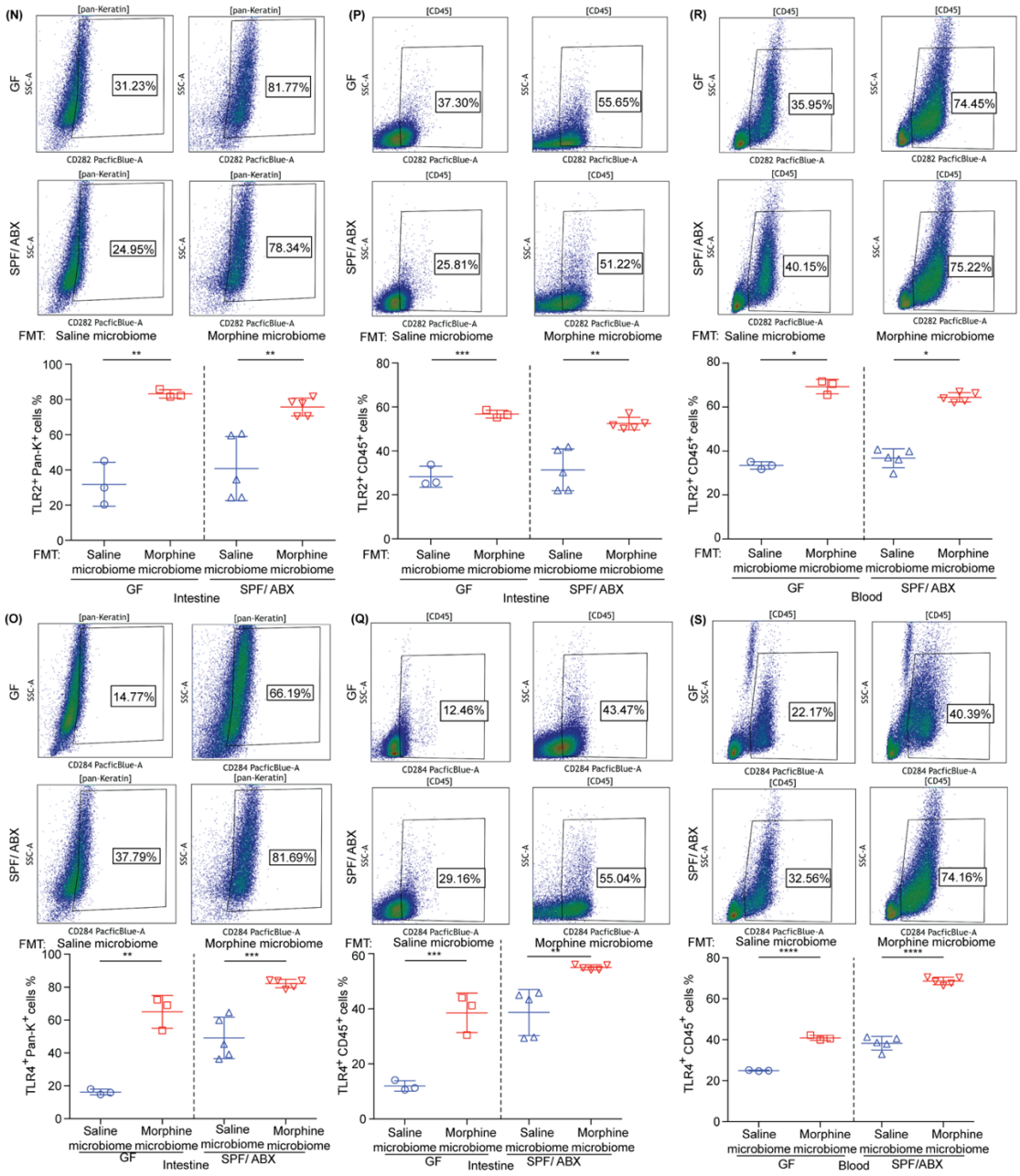


Fig. 3.4: Morphine regulates gut and systemic TLR2 and TLR4 expression through gut microbiome. (A) The 355-bp amplicon is specific for gram-positive bacteria and the 985-bp amplicon is specific for gram-negative bacteria. Lane 1 is DNA ladder. Lane 2-9 is bacteria DNA from liver homogenates from saline-treated mice; lane 10-17 is bacteria DNA from liver homogenates from morphine-treated mice; lane 18 is *C. Rodentium* for gram-negative bacteria as a positive control; lane 19 is *E. Faecalis* for gram-positive bacteria as a positive control; lane 20 is purified water for negative

control. (B-G) The up-regulation of TLR2 and TLR4 expression by morphine was suppressed in GF and ABX mice. n_{GF}=3; n_{SPF}=12-15. (H-M) Morphine induced TLR2 and TLR4 expression in GF mice following gavaging with naïve mouse microbiota. n_{GF}=3. (B-G) Data were analyzed by one-way ANOVA followed by Bonferroni's multiple comparisons test. (N-S) The microbiota FMT from morphine-tolerant mice alone increased TLR2 and TLR4 expression in GF and ABX mice. n_{GF}=3. n_{ABX}=5. Two-tailed student's t-test was used for statistical analysis.

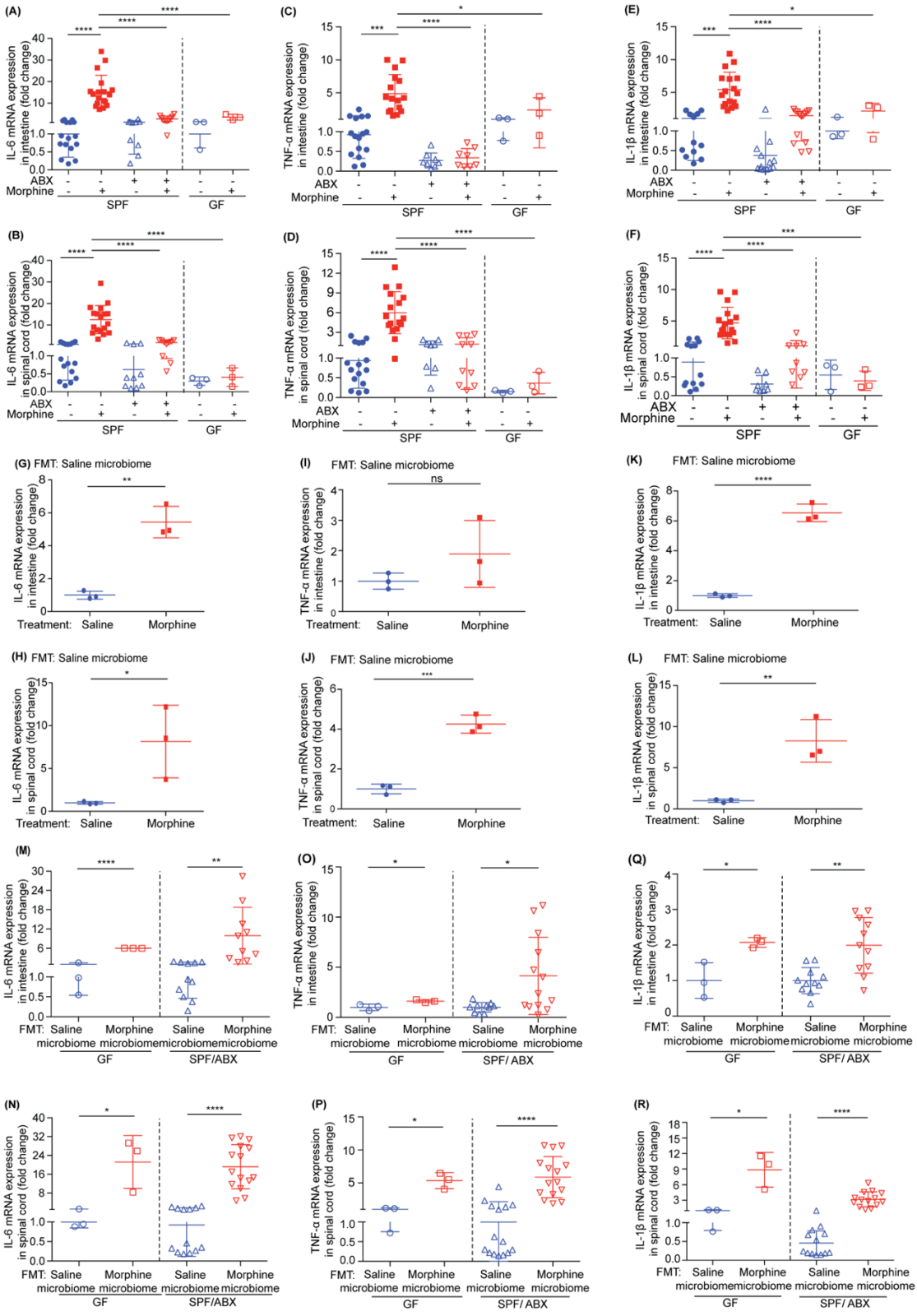


Fig. 3.5: Gut microbiome is essential for morphine-induced chronic systemic inflammation. (A-F) IL-6, TNFα, and IL-1β expression in morphine-treated GF and ABX mice was reduced when compared to WT controls. n_{GF}=3, n_{SPF}=10-17. (G-L)

Morphine effect on the expression of proinflammatory cytokines was restored after gut microbiota was reconstituted in GF mice. n_{GF}=3. (A-F) Data were analyzed using one-way ANOVA followed by Bonferroni's multiple comparisons test. (G-L) GF and ABX mice were gavaged with microbiota from either saline or morphine-tolerant mice. n_{GF}=3; $n_{SPF/ABX}$=13-15. Significance was tested by two-tailed student's t-test. (M-R) GF and ABX mice were gavaged with microbiota from either saline or morphine-tolerant mice. nGF=3; nSPF/ABX=13-15. Significance was tested by two-tailed student's t-test.

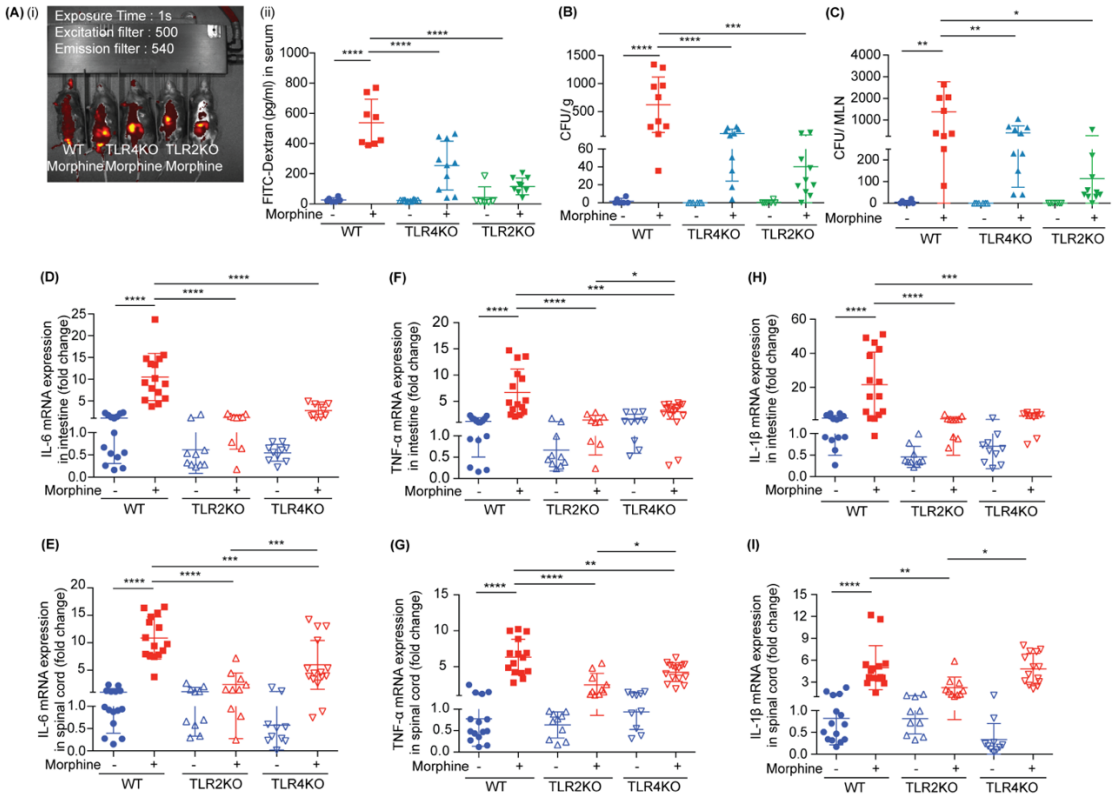


Fig. 3.6 Gut permeability, bacterial translocation and pro-inflammatory cytokine expression are attenuated in TLR2KO and TLR4KO mice compared to WT controls. (A) (i) Representative images and (ii) summary of fluorescent signal distribution in morphine-treated WT, TLR2KO and TLR4KO mice. n=6-10. (B, C) Visualization of Bacterial colonies in blood agar plates from liver and MLN homogenates. (D-I) IL-6, TNF-α, and IL-1β expressions were detected in WT, TLR2KO and TLR4KO mice. n=8-16.

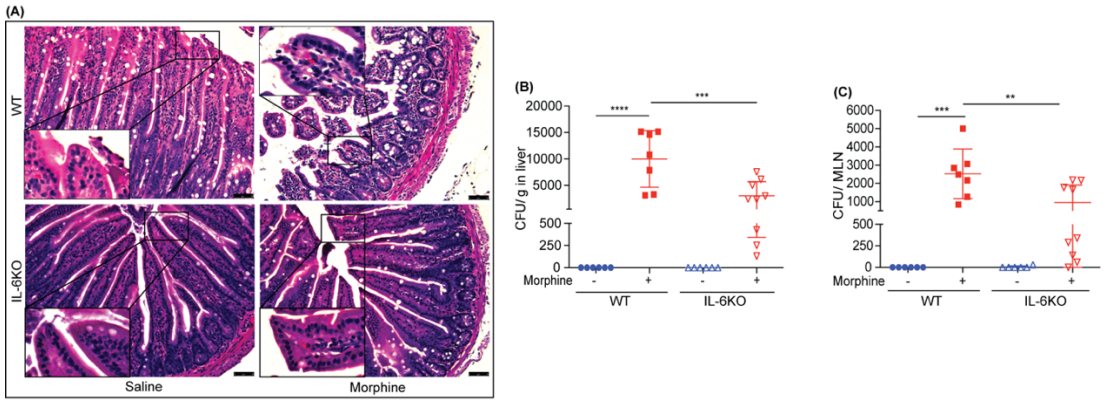


Fig. 3.7 Gut morphological damage and bacterial translocation are alleviated in IL-6KO mice. (*A*) Representative H&E stained intestinal sections from morphine treated-WT and IL-6KO mice. n=6. (*B*, *C*) The liver and MLN homogenates from WT and IL6KO mice were plated on blood agar plates overnight. Bacterial colonies were counted (colony forming unit). n=6-9. One-way ANOVA followed by Bonferroni's multiple comparisons test was used for statistical analysis. *, $p<0.05$; **, $p<0.01$; ***$p<0.001$; ****$p<0.0001$. Mean ± SD.

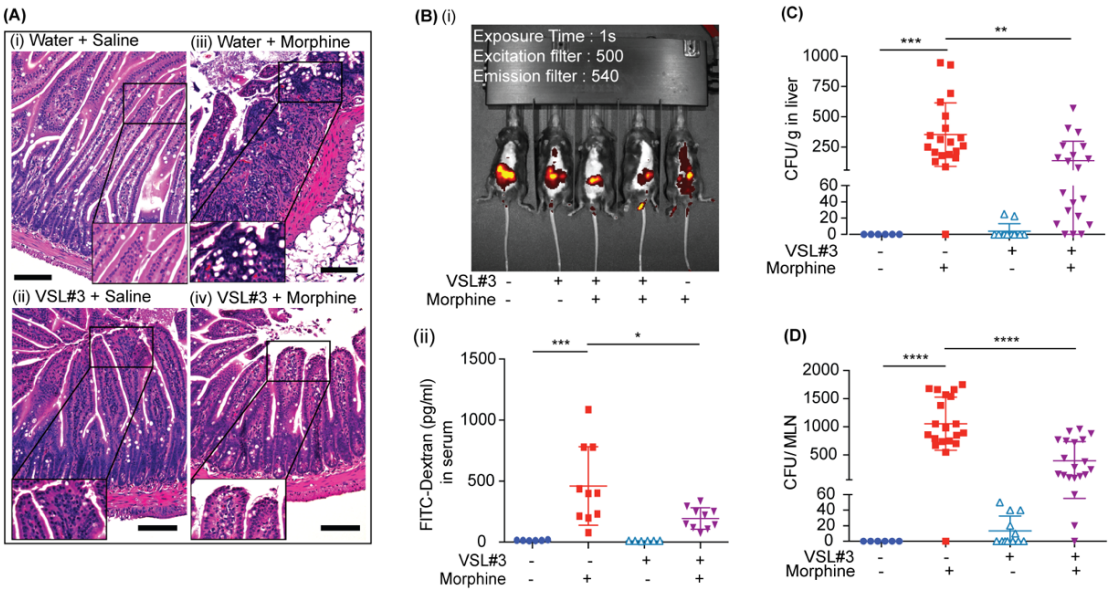


Fig. 3.8 Probiotics VSL#3 pre-treatment reverses morphine-induced gut pathology. (A) Representative H&E stained intestinal sections from different treatment groups. n=6-10. (B) (i) Representative figure of florescent signal distribution and (ii) summary of serum fluorescein isothiocyanate-dextran concentration from different treatment groups. n=6-10. (C, D) CFU from liver homogenate of each mouse in different treatment groups. n=6-20.

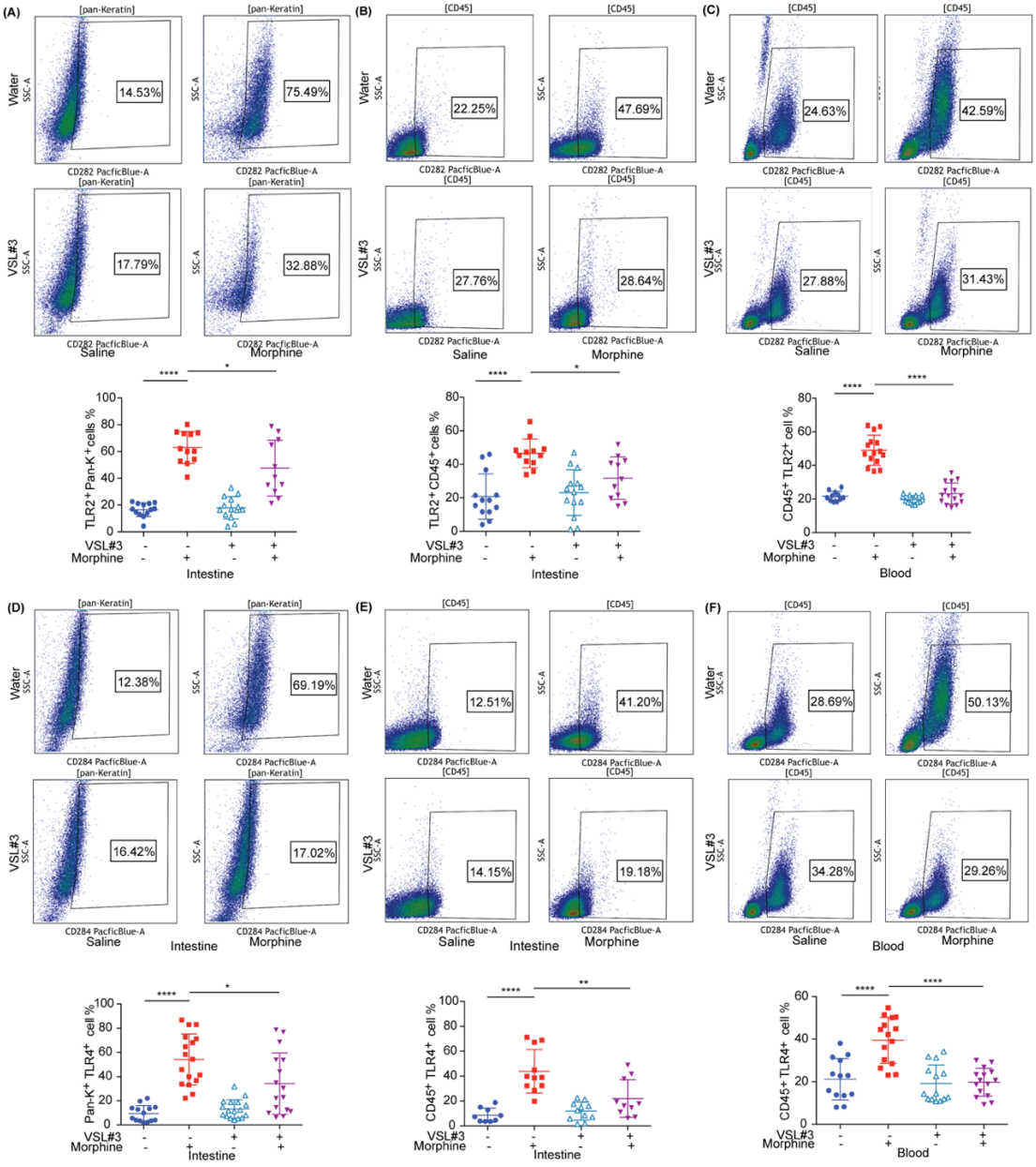


Fig. 3.9 Probiotics pre-treatment inhibits TLR2 and TLR4 up-regulation by morphine on epithelial and immune cells. n=11-14. The results were analyzed by one-way ANOVA followed by Bonferroni's multiple comparisons test. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****$p < 0.0001$. Mean ± SD.

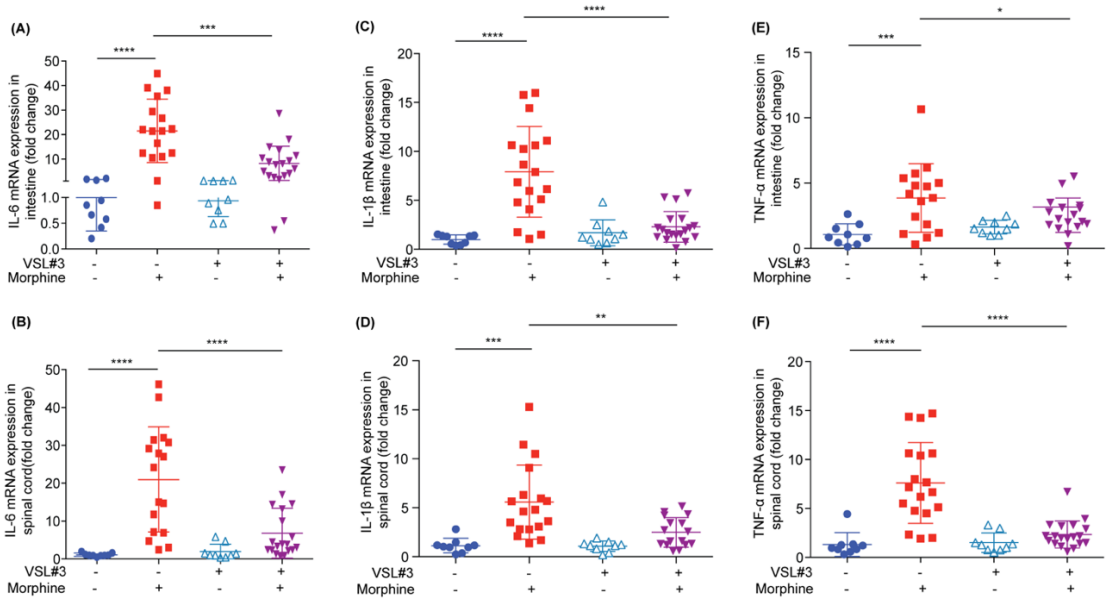


Fig. 3.10 Probiotics pretreatment attenuated morphine-induced proinflammatory cytokines expression. (A-F) RT-PCR of the IL-6, TNF-α, and IL-1β gene expression. n=9-19. One-way ANOVA followed by Bonferroni's multiple comparisons test was used to analyze the data. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p< 0.0001$. Mean ± SD.

Table 3.1: Inflammation induced by morphine tolerance was alleviated in SPF/ABX and GF mice.

Mice		SPF mice				GF mice	
Tissue		Treatment					
	Pro-inflammatory cytokines (pg/μg)	Water + Saline	ABX + Saline	Water + Morphine	ABX + Morphine	Saline	Morphine
Brain	IL-6	0.0755±0.0293***	0.0583±0.0364***	0.3743±0.1707	0.1499±0.0826**	0.0088±0.0055**	0.039±0.0019**
	IL-1β	0.0721±0.0616***	0.0463±0.0315****	0.3921±0.2102	0.0789±0.0541***	0.0194±0.0078****	0.0252±0.0098****
	TNFα	0.1222±0.0540****	0.1298±0.0439****	0.4263±0.1091	0.1790±0.0949****	0.0188±0.0036****	0.0234±0.0059****
Liver	IL-6	0.0420±0.0263***	0.0075±0.0074****	0.1112±0.0552	0.1076±0.0781***	0.0301±0.0206**	0.0393±0.0322*
	IL-1β	0.0373±0.0310**	0.0241±0.0169***	0.1180±0.0463	0.0621±0.0496*	0.0204±0.0286	0.0442±0.0365
	TNFα	0.0748±0.0388**	0.0422±0.0324****	0.2804±0.01909	0.1252±0.1252***	0.058.8±0.0346**	0.1138±0.0765*
MLN	IL-6	0.0158±0.0126****	0.0682±0.065.6*****	0.3625±0.1913	0.0327±0.0353****	0.0204±0.0044***	0.0123±0.0035***
	IL-1β	0.0402±0.0183***	0.0404±0.0258****	0.1668±0.0598	0.0589±0.0455****	0.0404±0.0055**	0.0272±0.0055**
	TNFα	0.0440±0.0348***	0.0382±0.0205****	0.1393±0.0643	0.028.1±0.0226****	0.0364±0.0088***	0.0388±0.0081***

IL-6, IL-1β and TNFα concentrations in the homogenates of brain, liver and MLN from SPF and GF mice were determined by ELISA. n_{WT}=6-10, n_{GF}=3. Data show Mean±SD. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$. Water+Morphine treatment group was used for statistical comparison. Significance was calculated by one-way ANOVA followed by Bonferroni's multiple comparisons test.

Table 3.2: After FMT with naïve SPF microbiota, GF mice displayed higher expression of cytokines following morphine treatment.

Mice		GF mice	
		Treatment	
	Pro-inflammatory cytokines (pg/μg)	Saline microbiome -Saline	Saline microbiome -Morphine
Brain	IL-6	0.0197±0.0107	0.1349±0.0694*
	IL-1β	0.0452±0.0164	0.2992±0.1081*
	TNFα	0.0238±0.0052	0.3132±0.1444*
Liver	IL-6	0.0566±0.0098	0.4967±0.2318*
	IL-1β	0.0756±0.0635	0.8375±0.4089*
	TNFα	0.1071±19.8	0.654±0.3056*
MLN	IL-6	0.0483±0.0310	0.3020±0.0945*
	IL-1β	0.1094±0.0724	0.5588±0.2297*
	TNFα	0.1283±0.0656	0.2132±0.0609***

After GF mice were transplanted with control microbiome (FMT), inflammatory cytokine levels were measured and compared to morphine-treated mice. Data show Mean ± SD. n_{GF}=3. The data were analyzed by one-way ANOVA followed by Bonferroni's multiple comparisons test. *, $p<0.05$; ***, $p<0.001$.

Table 3.3: Morphine microbiome FMT alone induced inflammation without exposure to morphine.

Mice		SPF mice		GF mice	
		Treatment			
	Pro-inflammatory cytokines (pg/μg)	Saline microbiome	Morphine microbiome	Saline microbiome	Morphine microbiome
Brain	IL-6	0.0164±0.0102	0.1561±0.0612****	0.0187±0.0115	0.1893±0.0754$^{\#}$
	IL-1β	0.0461±0.0221	0.1789±0.0657****	0.0465±0.0327	0.4851±0.1374$^{\#\#}$
	TNFα	0.0508±0.0290	0.3148±0.095.2*****	0.0721±0.0611	0.3945±0.0541$^{\#\#}$
Liver	IL-6	0.0304±0.0197	0.2281±0.1279***	0.0359±0.0081	0.2868±0.1354$^{\#}$
	IL-1β	0.026.9±0.0138	0.3248±0.1191****	0.0671±0.0293	0.3521±0.1260$^{\#}$
	TNFα	0.0369±0.0109	0.3241±0.1146****	0.0758±0.0165	0.03633±0.1558$^{\#}$
MLN	IL-6	0.0526±0.0329	0.292.2±0.1315*****	0.0204±0.0079	0.1493±0.0219$^{\#\#\#}$
	IL-1β	0.1272±0.1119	0.7111±0.3607****	0.0646±0.0308	0.1653±0.0255$^{\#}$
	TNFα	0.0462±0.0279	0.5247±0.2448****	0.0531±0.0116	0.2828±0.0402$^{\#\#\#}$

Saline microbiome group (FMT) was compared with morphine microbiome (FMT) in either SPF or GF mice. Data show Mean ± SD. n_{SPF}=10, n_{GF}=3. The data were analyzed by one-way ANOVA followed by Bonferroni's multiple comparisons test. *, $^{\#}$, $p<0.05$; **, $^{\#\#}$, $p<0.01$; ***, $^{\#\#\#\#}$, $p<0.001$; ****, $^{\#\#\#\#}$, $p<0.0001$

Table 3.4: Inflammation induced by morphine tolerance was attenuated in TLR2KO and TLR4KO mice.

Mice		WT mice		TLR2KO mice		TLR4KO mice	
Tissue		Treatment					
	Pro-inflammatory cytokines (pg/μg)	Saline	Morphine	Saline	Morphine	Saline	Morphine
Brain	IL-6	0.0192 ±0.0091****	0.1992±0.0638	0.0178±0.0041****	0.0210±0.0079****	0.0112 ±0.0055****	0.0159±0.0053****
	IL-1β	0.0403 ±0.0105****	0.2445±0.1150	0.0464±0.0135****	0.0596±0.0297****	0.0200 ±0.0119****	0.0376±0.0310****
	TNFα	0.0364 ±0.0134****	0.3071±0.0333	0.0430±0.0173 ****	0.0555±0.0269****	0.0070 ±0.0049****	0.0476±0.0168****
Liver	IL-6	0.0110 ±0.0044****	0.0623±0.0315	0.0114±0.0039****	0.0180±0.0093****	0.0136 ±0.0066****	0.0236±0.0145***
	IL-1β	0.0351 ±0.0175****	0.1515±0.0490	0.0160±0.0101****	0.0209±0.0063****	0.0298 ±0.0502****	0.0873±0.0520
	TNFα	0.0217 ±0.0180***	0.0873±0.0467	0.0080±0.0029****	0.0291±0.0215***	0.0300 ±0.0283****	0.0500±0.0221
MLN	IL-6	0.0177 ±0.0067****	0.1172±0.0493	0.0069±0.0051****	0.0322±0.0189****	0.0101 ±0.0055****	0.0189±0.0114****
	IL-1β	0.0170 ±0.0055****	0.0959±0.0217	0.0182±0.0089****	0.0459±0.0319**	0.0187 ±0.0101****	0.0682±0.0526*
	TNFα	0.0267 ±0.0158****	0.2458±0.1212	0.0147±0.0160****	0.0542±0.0310****	0.0406 ±0.0775****	0.0473±0.0196****

Inflammatory cytokine levels were measured in tissue homogenates. Data are represented as Mean ± SD. n_{WT}=7-10, n_{TLR2KO}=8-12, n_{TLR4KO}=8-12. One-way ANOVA followed by Bonferroni's multiple comparisons test was used for statistical analyses. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.

Table 5: Probiotics pre-treatment attenuated morphine tolerance-induced inflammation.

Tissue		Treatment			
	Pro-inflammatory cytokines (pg/μg)	Sham + Saline	VSL#3 + Saline	Sham + Morphine	VSL#3 + Morphine
Brain	IL-6	0.0202±0.0094****	0.239±0.0075****	0.1680±0.0896	0.0502±0.0725****
	IL-1β	0.0477±0.0119****	0.0389±0.0176****	0.2953±0.1089	0.1161±0.0980****
	TNFα	0.0343±0.0178****	0.0313±0.0160****	0.1969±0.0829	0.0622±0.0539****
Liver	IL-6	0.0144±0.0118**	0.0099±0.0039***	0.0438±0.0238	0.0228±0.0093*
	IL-1β	0.0981±0.0365****	0.0851±0.0236****	0.4084±0.1384	0.1422±0.1382****
	TNFα	0.0271±0.0229****	0.0256±0.0160****	0.1026±0.0305	0.0994±0.0723**#
MLN	IL-6	0.0196±0.0114****	0.0331±0.0314****	0.1110±0.0645	0.0279±0.0132****
	IL-1β	0.0280±0.0118***	0.0281±0.0134***	0.1212±0.0408	0.0739±0.0499*
	TNFα	0.0318±0.0236***	0.0345±0.0212***	0.1306±0.0626	0.0579±0.0325**

Cytokine levels from control treated animals (gavaged with water) and morphine treated animals were compared to all the other treatment groups. Data show Mean ± SD. n=5-12. One-way ANOVA followed by Bonferroni's multiple comparisons test was used. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$. Cytokine levels from control treated animals (gavaged with water) and treated with saline were compared to VSL#3 + Morphine. #$p<0.05$.

CHAPTER 4

THE LIMITATIONS OF THE STUDY AND FUTURE PLANS

In this study, we focused on the contribution of peripheral mechanisms to morphine-induced tolerance. We discovered that microbial dysbiosis, bacterial translocation, intestinal and systemic TLR2 and TLR4 activation, and inflammation as mediators of morphine analgesic tolerance. However, there are some limitations in our studies in their current form.

First of all, we emphasized cytokines, which augmented by morphine-induced dysbiosis, contributing to neuroinflammation and morphine tolerance. However, the morphine tolerance involves multiple mechanisms including OPRM1 signaling pathway, NMDA receptor system, morphine pharmacokinetics/pharmacodynamic, activation of microglia and astrocytes, interplay of different opioid receptors, other than neuroinflammation and peripheral inflammation. Our studies did not investigate whether manipulation of microbiota influences OPRM1 expression and its signaling pathway. As mentioned previously, *L. acidophilus* and *L. salivarius* induced an increased expression of OPRM1 mRNA in epithelial cells(184). It is expected that VSL#3 administration might increase OPRM1 expression in the gut (either epithelial cells or immune cells), and more morphine molecules could bind to the receptors, thus attenuating morphine tolerance. The FMT from morphine tolerant mice might decrease OPRM1 expression, thus accelerating morphine tolerance.

In our studies, we focused on the role of gut microbiota in morphine analgesic tolerance. The gut microbiota in TLR2KO mice were found to be resistant to chronic morphine

treatment. To further prove the role of gut microbiota on morphine tolerance, we plan on performing FMT of microbiota from TLR2KO mice or WT mice into GF mice and detect the generation of morphine tolerance. We hypothesize that chronic morphine treatment will not alter the microbiota from TLR2KO mice in GF mice, thus displaying less tolerance than the GF mice FMT with WT mouse microbiota. These experiments will allow us to identify bacterial communities that confer resistance to morphine-induced microbial change.

A number of studies associate disruption in commensal gut microbiota on CNS and behavioral changes mediated through vagus nerve, bacterial metabolites, and hormone, in addition to cytokines. The vagal system is crucial to gut-brain axis. *L. rhamnosus (JB-1)* reduced stress-induced corticosterone and anxiety- and depression-related behavior through modulating GABA receptors, but these changes were abolished in vagotomized mice(188). On the contrary, another study showed that when mice were infected with *T. muris*, the vagotomized mice displayed same anxiety-like behavior as sham infected mice without vagotomy(189). These findings indicate that vagal system might be one of the pathways how dysbiosis or gut inflammation affects behavior. There are limited studies investigated vagal nerve involvement in morphine tolerant rodent models. The limitations of our studies are that we have not studied if morphine-induced dysbiosis has an impact on vagal nerve function and its contribution to morphine tolerance.

Our studies suggest that chronic morphine treatment depletes beneficial bacteria such as *Lactobacillus* and *Bifidobacteria* and facilitates the expansion of pathobiome. However, our analysis is limited to the genus level because our sequencing method is

based on 16S rRNA with a single marker gene. The bacterial species in *Lactobacillus* and *Bifidobacteria* genus, which have neuroprotective properties against morphine-induced neuroinflammation and analgesic tolerance, have not been exploited. In addition, our studies discuss the role of dysbiosis in regulation of gastrointestinal inflammation and its impact on neuroinflammation in morphine tolerant model. However, we have not systematically analyzed the functional metagenomics of the altered microbiome in detail. The analysis of functional profile of the microbial communities will determine how bacterial gene content regulates the host. Preliminary studies have shown chronic morphine treatment increased functional gene profile related to cell motility, human diseases such as cancer, neurodegenerative disease and cardiovascular disease and decreased genes related to metabolism of terpenoids and polyketides, nucleotide metabolism, amino acid metabolism, xenobiotics biodegradation and metabolism and carbohydrate metabolism (Fig. 4A). The VSL#3, which replenished the missing bacteria, rebalanced these functions (Fig. 4B). However, the single marker gene of 16S rRNA is susceptible to provide us biased results.

To solve these two potential problems, whole-metagenome shotgun sequencing should be applied. The non-targeted sequencing of DNA fragments will decipher taxonomy to species level or even strain level. Analyses based on shotgun deep sequencing will provide direct and precise functional inference. Furthermore, to further prove the predictive chronic morphine-induced alterations, the meta-metabolome analysis such as liquid chromatography-mass spectrometry will be carried out.

In addition, we suggested that TLR2 and TLR4 are components of the gut-brain axis that contribute to morphine tolerance. However, the roles of TLR2 and TLR4 in

morphine analgesic tolerance have not been fully delineated. Previous studies showed that TLR2 and TLR4 expression were upregulated in microglia and gut epithelial cells(59)(163). TLR2 and TLR4 protect morphine-induced gut epithelial tight junction disruption in a MLCK-dependent manner(59). In our studies, TLR2KO and TLR4KO mice have protective properties against neuroinflammation following chronic morphine challenge. TLR2 and TLR4 have been shown to play critical roles in morphine dependence, withdrawal, and tolerance. However, there are limited studies on the interaction between TLR2/4 and OPRM1 signaling pathways. Studies have shown that both chronic morphine treatment and activation of TLR2/4 share the downstream signaling pathways of MAPK, NF-κB, AP-1 and CREB(102)(190). We hypothesize that morphine activates OPRM1 and might increase the expression or activate TLR2/4 downstream pathways through their shared signaling pathway. The activation of TLR2/4 initiates the production of pro-inflammatory cytokines. Proinflammatory cytokines such as IL-6 also share MAPK signaling pathway with OPRM1 signaling pathways(191). Additionally, studies have shown that morphine tolerance was attenuated by inhibiting p38 MAPK in activated spinal microglia(158). The interaction among all the signaling pathways exacerbated chronic morphine-induced adverse effects. Additionally, the PU.1 that is activated by TLR4 signaling was found to bind to the promoter of OPRM1 gene and transcriptionally regulate its expression(191). We hypothesize TLR2/4 or IL-6 might regulate PU.1 binding to OPRM1 gene to affect the OPRM1 expression and activation. We could answer this question by comparing OPRM1 expression in WT mice and TLR2KO, TLR4KO and IL-6KO mice. Moreover, cytokines or chemokines might regulate the neurotransmitters and their receptors that could play a central role in morphine tolerance. It is hypothesized that IL-6 might

increase the intracellular calcium responses to NMDA by increasing intracellular calcium signals, therefore sensitizing neuronal response to xenobiotics(192).

Our studies have shown an increase in TLR2 and TLR4 expression on immune cells and gut epithelial cells. Therefore, morphine tolerance attenuation is observed in TLR2KO and TLR4KO mice. TLR2/4 expression on which cells contribute to morphine tolerance are of great interest for the development of tolerance. To further explore these questions, two models can be used.

One model is to use bone marrow transplantation. There is evidence to show that peripheral immune cells have a causative effect on CNS. Grace et al. have reported that transplantation of splenocytes from a rat donor with chronic constriction injury that displayed higher allodynia to a recipient rat potentiated the recipient's low allodynia(193). Similarly, we can transplant the bone marrow of WT mice into TLR2KO or TLR4KO mice and vice versa. If the TLR on epithelial cells are responsible for the morphine-induced dysbiosis and tolerance, despite bone marrow transplantation from WT mice, the gut microbiota will be resistant to chronic morphine treatment. Moreover, the dysbiosis of WT mice, in spite of bone marrow transplantation of TLR2KO or TLR4KO mice, will be induced by morphine. However, if the TLR on immune cells are responsible for the morphine-induced microbial alteration and behavior changes, the dysbiosis will be manifested in TLR2KO and TLR4KO mice after bone marrow transplantation of WT mice. The WT with TLR2KO or TLR4KO bone marrow transplantation will display attenuated morphine tolerance with less dysbiosis.

Another model that will allow us to discriminate the cell type that is responsible for morphine-induced tolerance is the use of cre-lox mice mediated conditional knockout. Conditional TLR deletion on epithelial cells have been generated by breeding floxed TLR ($TLR^{fl/fl}$) with villin-cre (Vil-Cre) mice. If TLR on epithelial cells are responsible for morphine-induced dysbiosis and analgesic tolerance, the conditional TLR knockout mice will exhibit similar response as conventional TLRKO mice. Moreover, to demonstrate whether TLR on immune cells is the primary cause of morphine-induced pathological changes in the gut and behavior, the floxed TLR ($TLR^{fl/fl}$) can be crossed with lysosome-cre (lysM-Cre) mice to generate conditional TLR knockout on myeloid cells. If TLR on immune cells contributes to morphine-induced dysbiosis and analgesic tolerance, the conditional TLR knockout mice will resemble the microbiota profile as conventional TLRKO mice. These studies can delineate how peripheral TLR2 and TLR4 regulate CNS, thus contributing to morphine analgesic tolerance.

In our studies, we demonstrated that TLR2 and TLR4 are crucial elements to morphine-induced analgesic tolerance. As previously mentioned, LPS-RS, a TLR4 antagonist, prevented both the development of analgesic tolerance and the acquisition and maintenance of morphine-induced CPP(90). Simultaneously, HKLM, a TLR2 agonist, induced lasting tactile allodynia in mice(95). Therefore, we propose that TLR2 and TLR4 antagonists are potential agents to inhibit chronic morphine-induced behavioral changes. Small molecule drug screening could be undertaken to identify novel drugs to inhibit the activation and expression of TLR2 and TLR4.

In our studies, we propose IL-6 as a downstream factor of TLR2 and TLR4 activation that can contribute to morphine tolerance. However, our results show only partial attenuation of morphine tolerance in the IL-6KO mice. This suggests that other factors may be involved in morphine tolerance, including cytokines/chemokines such as IL-1β, TNFα, CXCL1, IL-10, CXCL12 and other peripheral factors such as complement factor 5 receptor (C5aR), tissue plasminogen activator (tPA), neuronal matrix metalloproteinase 9 (MMP9), and neuronal nitric oxide synthase(80)(82)(83)(84)(85)(86). Studies have confirmed the presence of additional peripheral mechanisms affecting morphine tolerance. For example, morphine tolerance could be abolished by blockade of peripheral MOR on DRG by methylnaltrexone bromide, or blockade of JAK2/STAT3 pathway by regulating miR-375, or activation of Mrg3C receptor in DRG(194)(195)(196). Taken together, these studies have indicated that peripheral MOR activation plays a crucial role in morphine tolerance, and therapeutics targeted at inhibiting peripheral MOR may prolong morphine's efficacy at central analgesic sites.

In our studies, we focused on increasing cytokine levels in CNS in morphine tolerant mice. Additionally, many investigators have clearly demonstrated that cytokines and chemokines are upregulated by activated microglia and astrocytes and infiltrating immune cells(79). Future studies will characterize the role of microglia and astrocytes in morphine analgesic tolerance will be determined by detecting Ionized calcium binding adaptor molecule 1(Iba1) as a marker of microglia and glial fibrillar acidic protein (GFAP) as a marker of astrocyte(197). The morphological changes and activities of non-neuronal cells will be determined in morphine-treated GF mice, ABX mice, probiotics-treated mice, TLR2KO, TLR4KO and IL6KO mice and their controls.

To detect the role of TLR2 and TLR4 on microglia or astrocyte on analgesic tolerance, Cx3cr1-cre mice or Aldh1l1-cre mice can be crossed with $TLR^{fl/fl}$ mice to specifically delete TLR on microglia or astrocytes(198)(199).

In addition to changes on immune cells, we speculate that alterations on morphine concentrations in CNS might contribute to analgesic tolerance. To determine whether morphine-induced dysbiosis affects morphine concentrations in CNS, we will administer morphine-D_3 in naïve and chronic morphine-treated mice. Then the concentration of morphine-D_3, and its metabolites morphine-D_3-6-β-D-glucuronide and morphine-D_3-3-β-D-glucuronide will be determined in the plasma, CNS and intestinal contents by liquid chromatography–mass spectrometry (LC-MS). We hypothesized that there will be an accumulation of morphine-D_3-6-β-D-glucuronide and morphine-D_3-3-β-D-glucuronide in the mouse intestinal contents and reduced morphine-D_3 concentration in mouse plasma and CNS in chronic morphine-treated mice compared to morphine-naïve mice. It is speculated that β-glucuronidase activity is significantly decreased due to beneficial bacteria depletion in morphine tolerant mice. Therefore the activity and expression of β-glucuronidase will be compared in morphine naïve mice and chronic morphine-treated mice.

Both our studies and Taylor et al. demonstrated gut dysbiosis in morphine-treated mice(79)(118). However, the fundamental question of how morphine induces gut dysbiosis has not been clearly elucidated. Banerjee et al. showed morphine altered the cholesterol metabolism, converting cholesterol into coprostanol instead of primary bile acids(118). Consequently, less primary and secondary bile acids were detected in the intestine(60). Reduced bile acids in the intestine are associated with pathogenic

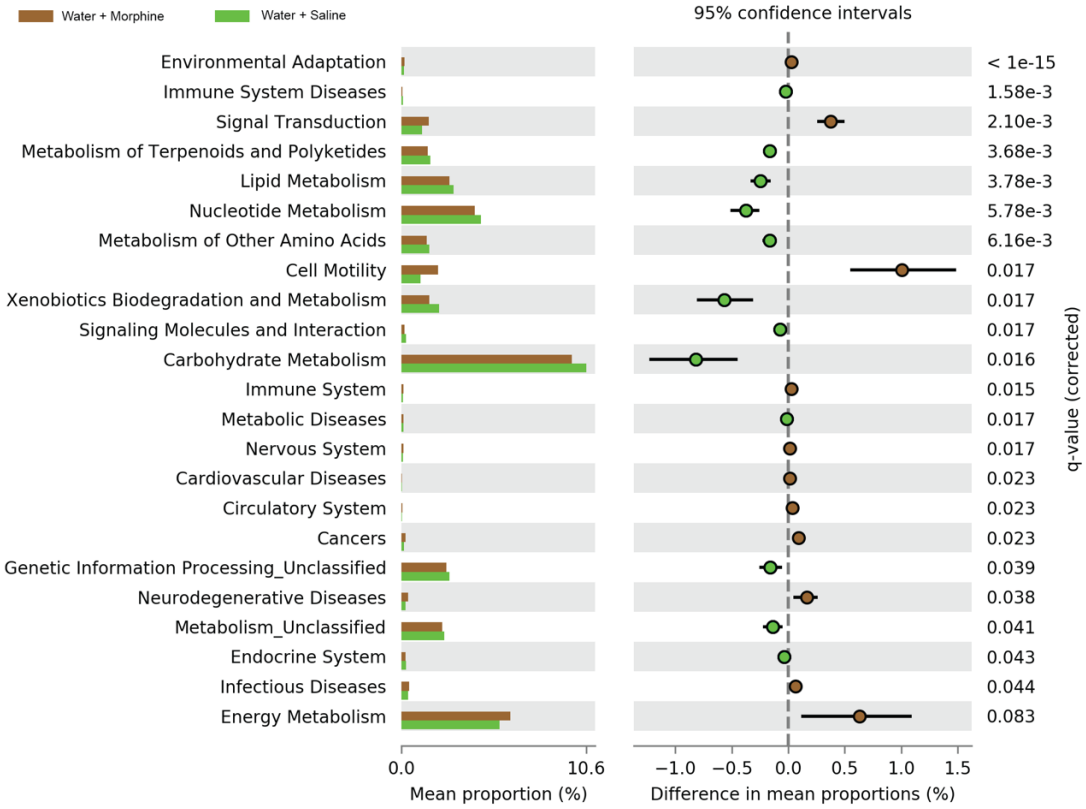
bacterial overgrowth and inflammation(200). Simutaneously, the morphine-induced deleterious intestinal environment suppresses the growth of beneficial bacteria with bile acid hydrolase (BSH) and β-glucuronidase activity(118). Therefore, imbalance between beneficial bacterial and pathogenic bacteria can alter primary and secondary bile acids in the gut, thus excerbating morphine-induced dysbiosis. However, the precise mechanisms of how morphine induces dysbiosis are not completely understood. Whether supplementation of bile acids or bile acid derivatives could attenuate morphine-induced dysbiosis and morphine-induced analgesic tolerance needs further investigation.

Additionally, we determined that morphine-induced gut dysbiosis regulates inflammation, contributing to analgesic tolerance. The immune system keeps a delicate balance by eliminating invading pathogens while maintaining self-toleance to symbiotic bacteria. The commensal bacteria induce regulatory T cells, regulate SIgA synthesis and diversity of antibody repertoires, and thereby promote immunological tolerance and preventing immune overreaction(201). Moreover, the gut barrier, mucus, and antimicrobial peptides shape and control the composition of enteric inhabitants(202). However, morphine-induced gut dysbiosis causes immune dysregulation by altering the equilibrium between immune tolerance and immune activation. Morphine induced-dysbiosis facilitates bacterial translocation. We hypothesize that due to acute morphine-induced immunosuppression(203), the translocated bacteria and bacterial components such as LPS, LTA and endotoxin are not eliminated properly and thereby heading to induce chronic inflammation. The chronic immune activation can damage tight junction proteins in the gut epithelial cells and cause more bacterial translocation and further excerbation of chronic inflammation,

contributing to morphine analgesic tolerance. However, detailed mechanistic studies on how morphine-induced dysbiosis abrogates immune tolerance warrents studies.

In conclusion, morphine tolerance is a complex phenomenon involving pathways at the molecular, cellular and systemic levels. At the molecular level, several mechanisms are to be explored: the interaction between OPRM1 and TLR2/4 and their shared downstream signaling pathways to elucidate how morphine activates and increases the expression of TLR2/4. Furthermore, how inflammatory cytokines contribute to morphine tolerance through regulating levels of neurotransmitters, their receptors and downstream signaling pathways are also areas of further investigation. At the cellular level, which cell type (epithelia, immune cells, microglia or astrocytes) contributes to morphine-induced dysbiosis and influences the behavioral changes are important area I am interested in. At the systemic level, concentration of morphine and its metabolites and other possible mediators (vagus afferents and microbiota metabolites) that modulate in gut-brain axis, in addition to cytokines needs to be explored.

(A)


Water + Morphine
Water + Saline
95% confidence intervals
Environmental Adaptation
Immune System Diseases
Signal Transduction
Metabolism of Terpenoids and Polyketides
Lipid Metabolism
Nucleotide Metabolism
Metabolism of Other Amino Acids
Cell Motility
Xenobiotics Biodegradation and Metabolism
Signaling Molecules and Interaction
Carbohydrate Metabolism
Immune System
Metabolic Diseases
Nervous System
Cardiovascular Diseases
Circulatory System
Cancers
Genetic Information Processing_Unclassified
Neurodegenerative Diseases
Metabolism_Unclassified
Endocrine System
Infectious Diseases
Energy Metabolism
< 1e-15
1.58e-3
2.10e-3
3.68e-3
3.78e-3
5.78e-3
6.16e-3
0.017
0.017
0.017
0.016
0.015
0.017
0.017
0.023
0.023
0.023
0.039
0.038
0.041
0.043
0.044
0.083
q-value (corrected)
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Mean proportion (%)
−1.0 −0.5 0.0 0.5 1.0 1.5
Difference in mean proportions (%)

(B)

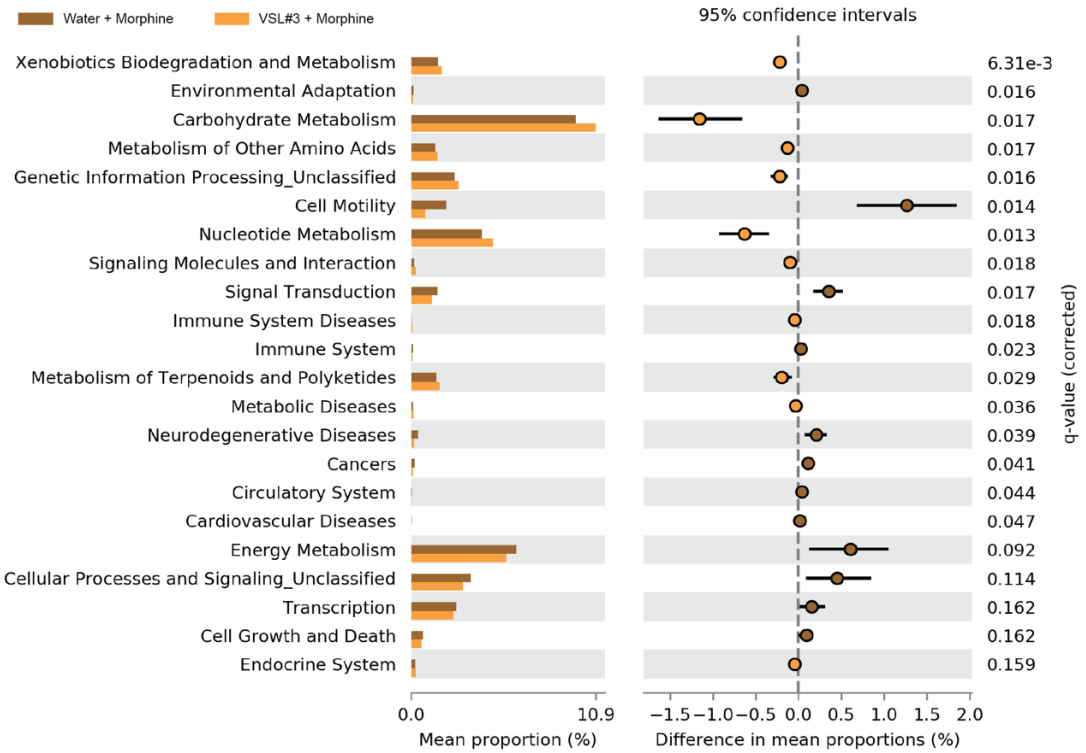
Water + Morphine
VSL#3 + Morphine
95% confidence intervals
Xenobiotics Biodegradation and Metabolism
Environmental Adaptation
Carbohydrate Metabolism
Metabolism of Other Amino Acids
Genetic Information Processing_Unclassified
Cell Motility
Nucleotide Metabolism
Signaling Molecules and Interaction
Signal Transduction
Immune System Diseases
Immune System
Metabolism of Terpenoids and Polyketides
Metabolic Diseases
Neurodegenerative Diseases
Cancers
Circulatory System
Cardiovascular Diseases
Energy Metabolism
Cellular Processes and Signaling_Unclassified
Transcription
Cell Growth and Death
Endocrine System
6.31e-3
0.016
0.017
0.017
0.016
0.014
0.013
0.018
0.017
0.018
0.023
0.029
0.036
0.039
0.041
0.044
0.047
0.092
0.114
0.162
0.162
0.159
q-value (corrected)
0.0
10.9
Mean proportion (%)
−1.5 −1.0 −0.5 0.0 0.5 1.0 1.5 2.0
Difference in mean proportions (%)

Figure 4.1 Gene contents inferred from the 16S ribosomal RNA counts based on Integrated Microbial Genomes (IMG) database reveal functional contents of mouse gut microbiota of Water+Saline and Water+Morphine treatment groups (A) and from Water+Morphine and VSL#3+Morphine (B), based on the KEGG functional modules. The relative abundance of functional contents was compared between the treated and control using Mann-Whitney U-test.

CHAPTER 5

CLINICAL RELEVANCE AND CONCLUSION

Morphine is a classical painkiller that was isolated from poppy straw of the opium poppy in 1803. Currently, opioids (oxycodone, methadone, morphine, hydrocodone and fentanyl) are still among top 10 drugs involved in drug overdose deaths(204). The "opioid crisis" related with misuse and addiction to opioids still affect public health and social and economic welfare. There are growing literature demonstrating gut microbiota alteration in substance abuse, including alcohol, tobacco, cocaine, hallucinogens (phencyclidine and ketamine), methamphetamine, 3,4-methylenedioxymethamphetamine (MDMA), morphine, heroin, marijuana(205). However, the precise mechanisms how microbiota modulate drug tolerance, dependence and addiction have not been clearly delineated. The gut-brain axis paves a new path to solve opioid crisis and drug abuse problem. Better understanding of the mechanism of microbiota-host interaction can help develop novel therapeutic strategy to solve the "opioid crisis".

Our study provides evidence for the role of the gut-immune-CNS axis in morphine analgesic tolerance. Chronic morphine use induces gut dysbiosis and initiates local inflammation through TLR2 and TLR4 activation. This results in increased expression of pro-inflammatory cytokines, which contributes to morphine tolerance, and acts as a feed-forward loop in aggravating dysbiosis, impairing gut integrity, leading to further bacterial translocation, thus exacerbating inflammation and sustaining morphine tolerance (Fig 5).

In our study, we use VSL#3 probiotics to restore morphine-induced dysbiosis, therefore

attenuating tolerance in a rodent model. Our study highlights the missing bacteria *Bifidobacterium* and *Lactobacillus* in morphine-induced behavior changes, which provide a new insight into their role in modulating morphine analgesic tolerance. More detailed studies are needed to detect the gut dysbiosis on opioid-dependent humans. Our studies in rodents can be used as a backdrop to design future human studies on how modulating gut microbiota can be exploited to prolong the efficacy of opioids as analgesic agents.

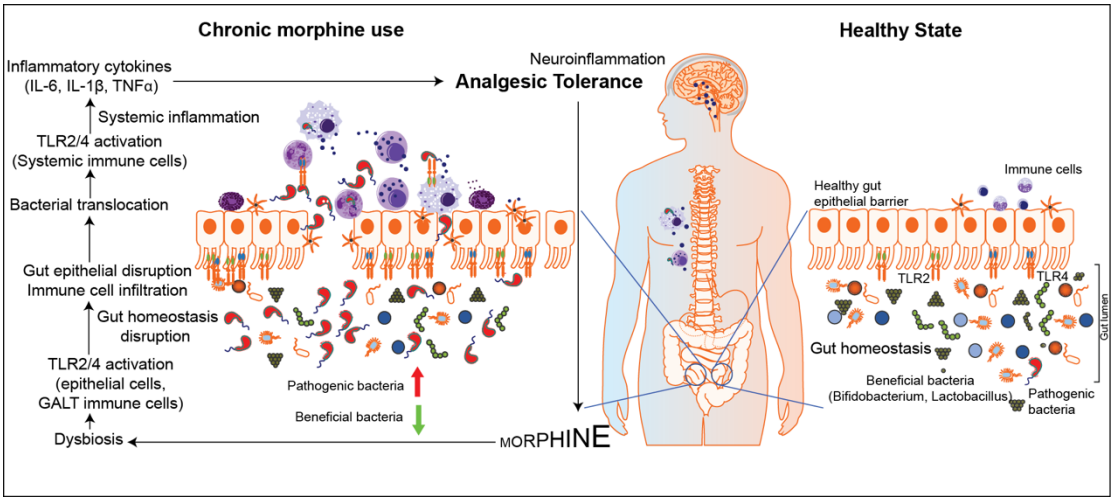

Figure 5.1 Schematic diagram shows the role of the gut-immune-brain axis in morphine tolerance. Chronic morphine use induces gut dysbiosis and initiates local inflammation through TLR2 and TLR4 activation. This results in increased expression of pro-inflammatory cytokines, contributes to morphine tolerance, and acts as a feed-forward loop in aggravating dysbiosis, impairing gut integrity, leading to further bacterial translocation, thus exacerbating inflammation and sustaining morphine tolerance.

LITERATURE CITED

1. Schroeder BO, Bäckhed F. Signals from the gut microbiota to distant organs in physiology and disease. *Nat Med* (2016) **22**:1079–1089. doi:10.1038/nm.4185
2. Cryan JF, Dinan TG. Mind-altering microorganisms: The impact of the gut microbiota on brain and behaviour. *Nat Rev Neurosci* (2012) **13**:701–712. doi:10.1038/nrn3346
3. Christensen EG. *Effect of gut microbiota on intestinal integrity*. (2014). Available at: http://www.food.dtu.dk/english/news/2014/05/phd-defence-on-bacteria-in-the-human-gut?id=9ebcdbff-2636-419d-9e3d-9068caea1b9b%0Ahttp://www.food.dtu.dk/english/news/2014/08/gut-microbiota-affects-intestinal-integrity?id=f1d16d54-1058-4152-8ce7-c573d582db57
4. Huttenhower C, Gevers D, Knight R, Abubucker S, Badger JH, Chinwalla AT, Creasy HH, Earl AM, Fitzgerald MG, Fulton RS, et al. Structure, function and diversity of the healthy human microbiome. *Nature* (2012) **486**:207–214. doi:10.1038/nature11234
5. Zeng MY, Inohara N, Nuñez G. Mechanisms of inflammation-driven bacterial dysbiosis in the gut. *Mucosal Immunol* (2017) **10**:18–26. doi:10.1038/mi.2016.75
6. Dai C, Zhao DH, Jiang M. VSL#3 probiotics regulate the intestinal epithelial barrier in vivo and in vitro via the p38 and ERK signaling pathways. *Int J Mol Med* (2012) **29**:202–208. doi:10.3892/ijmm.2011.839
7. Wahlström A, Sayin SI, Marschall HU, Bäckhed F. Intestinal Crosstalk between Bile Acids and Microbiota and Its Impact on Host Metabolism. *Cell Metab* (2016) **24**:41–50. doi:10.1016/j.cmet.2016.05.005

8. Damman CJ, Miller SI, Surawicz CM, Zisman TL. The microbiome and inflammatory bowel disease: Is there a therapeutic role for fecal microbiota transplantation. *Am J Gastroenterol* (2012) **107**:1452–1459. doi:10.1038/ajg.2012.93

9. Wang Z, Klipfell E, Bennett BJ, Koeth R, Levison BS, Dugar B, Feldstein AE, Britt EB, Fu X, Chung YM, et al. Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. *Nature* (2011) **472**:57–65. doi:10.1038/nature09922

10. Russell SL, Gold MJ, Hartmann M, Willing BP, Thorson L, Wlodarska M, Gill N, Blanchet MR, Mohn WW, McNagny KM, et al. Early life antibiotic-driven changes in microbiota enhance susceptibility to allergic asthma. *EMBO Rep* (2012) **13**:440–447. doi:10.1038/embor.2012.32

11. De Theije CGM, Wu J, Da Silva SL, Kamphuis PJ, Garssen J, Korte SM, Kraneveld AD. Pathways underlying the gut-to-brain connection in autism spectrum disorders as future targets for disease management. *Eur J Pharmacol* (2011) **668**:70–80. doi:10.1016/j.ejphar.2011.07.013

12. Stanley D, Mason LJ, MacKin KE, Srikhanta YN, Lyras D, Prakash MD, Nurgali K, Venegas A, Hill MD, Moore RJ, et al. Translocation and dissemination of commensal bacteria in post-stroke infection. *Nat Med* (2016) **22**:1277–1284. doi:10.1038/nm.4194

13. Brown K, Godovannyi A, Ma C, Zhang Y, Ahmadi-Vand Z, Dai C, Gorzelak MA, Chan Y, Chan JM, Lochner A, et al. Prolonged antibiotic treatment induces a diabetogenic intestinal microbiome that accelerates diabetes in NOD mice. *ISME J* (2016) **10**:321–332. doi:10.1038/ismej.2015.114

14. Hevia A, Milani C, López P, Cuervo A, Arboleya S, Duranti S, Turroni F,

González S, Suárez A, Gueimonde M, et al. Intestinal dysbiosis associated with systemic lupus erythematosus. *MBio* (2014) **5**:1–10. doi:10.1128/mBio.01548-14

15. Koeth RA, Wang Z, Levison BS, Buffa JA, Org E, Sheehy BT, Britt EB, Fu X, Wu Y, Li L, et al. Intestinal microbiota metabolism of l-carnitine, a nutrient in red meat, promotes atherosclerosis. *Nat Med* (2013) **19**:576–585. doi:10.1038/nm.3145
16. Stavrou G, Kotzampassi K. Gut microbiome, surgical complications and probiotics. *Ann Gastroenterol* (2017) **30**:45–53. doi:10.20524/aog.2016.0086
17. Lim SL, Rodriguez-Ortiz CJ, Kitazawa M. Infection, systemic inflammation, and Alzheimer's disease. *Microbes Infect* (2015) **17**:549–556. doi:10.1016/j.micinf.2015.04.004
18. Sampson TR, Debelius JW, Thron T, Janssen S, Shastri GG, Ilhan ZE, Challis C, Schretter CE, Rocha S, Gradinaru V, et al. Gut Microbiota Regulate Motor Deficits and Neuroinflammation in a Model of Parkinson's Disease. *Cell* (2016) **167**:1469-1480.e12. doi:10.1016/j.cell.2016.11.018
19. Breit S, Kupferberg A, Rogler G, Hasler G. Vagus nerve as modulator of the brain-gut axis in psychiatric and inflammatory disorders. *Front Psychiatry* (2018) **9**: doi:10.3389/fpsyt.2018.00044
20. Al-Asmakh M, Zadjali F. Use of germ-free animal models in microbiota-related research. *J Microbiol Biotechnol* (2015) **25**:1583–1588. doi:10.4014/jmb.1501.01039
21. Galland L. The Gut Microbiome and the Brain. *J Med Food* (2014) **17**:1261–1272. doi:10.1089/jmf.2014.7000
22. Sampson TR, Mazmanian SK. Control of brain development, function, and

behavior by the microbiome. *Cell Host Microbe* (2015) **17**:565–576. doi:10.1016/j.chom.2015.04.011

23. Matsumoto M, Kibe R, Ooga T, Aiba Y, Kurihara S, Sawaki E, Koga Y, Benno Y. Impact of intestinal microbiota on intestinal luminal metabolome. *Sci Rep* (2012) **2**:1–10. doi:10.1038/srep00233

24. Velagapudi VR, Hezaveh R, Reigstad CS, Gopalacharyulu P, Yetukuri L, Islam S, Felin J, Perkins R, Borén J, Orešič M, et al. The gut microbiota modulates host energy and lipid metabolism in mice. *J Lipid Res* (2010) **51**:1101–1112. doi:10.1194/jlr.m002774

25. Diaz Heijtz R, Wang S, Anuar F, Qian Y, Björkholm B, Samuelsson A, Hibberd ML, Forssberg H, Pettersson S. Normal gut microbiota modulates brain development and behavior. *Proc Natl Acad Sci U S A* (2011) **108**:3047–52. doi:10.1073/pnas.1010529108

26. Liu YW, Liu WH, Wu CC, Juan YC, Wu YC, Tsai HP, Wang S, Tsai YC. Psychotropic effects of Lactobacillus plantarum PS128 in early life-stressed and naïve adult mice. *Brain Res* (2016) **1631**:1–12. doi:10.1016/j.brainres.2015.11.018

27. Desbonnet L, Garrett L, Clarke G, Bienenstock J, Dinan TG. The probiotic Bifidobacteria infantis: An assessment of potential antidepressant properties in the rat. *J Psychiatr Res* (2008) **43**:164–174. doi:10.1016/j.jpsychires.2008.03.009

28. Burokas A, Arboleya S, Moloney RD, Peterson VL, Murphy K, Clarke G, Stanton C, Dinan TG, Cryan JF. Targeting the Microbiota-Gut-Brain Axis: Prebiotics Have Anxiolytic and Antidepressant-like Effects and Reverse the Impact of Chronic Stress in Mice. *Biol Psychiatry* (2017) **82**:472–487.

doi:10.1016/j.biopsych.2016.12.031

29. Pan W, Stone KP, Hsuchou H, Manda VK, Zhang Y, Kastin AJ. Cytokine Signaling Modulates BBB Function. *Curr Pharm Des* (2014) **17**:3729–3740.

30. de Theije CGM, Wopereis H, Ramadan M, van Eijndthoven T, Lambert J, Knol J, Garssen J, Kraneveld AD, Oozeer R. Altered gut microbiota and activity in a murine model of autism spectrum disorders. *Brain Behav Immun* (2014) **37**:197–206. doi:10.1016/j.bbi.2013.12.005

31. Daulatzai MA. Non-celiac gluten sensitivity triggers gut dysbiosis, neuroinflammation, gut-brain axis dysfunction, and vulnerability for dementia. *CNS Neurol Disord Drug Targets* (2015) **14**:110–31. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25642988

32. Lee YK, Menezes JS, Umesaki Y, Mazmanian SK. Proinflammatory T-cell responses to gut microbiota promote experimental autoimmune encephalomyelitis. *Proc Natl Acad Sci* (2011) **108**:4615–4622. doi:10.1073/pnas.1000082107

33. Ochoa-Reparaz J, Mielcarz DW, Ditrio LE, Burroughs AR, Foureau DM, Haque-Begum S, Kasper LH. Role of Gut Commensal Microflora in the Development of Experimental Autoimmune Encephalomyelitis. *J Immunol* (2009) **183**:6041–6050. doi:10.4049/jimmunol.0900747

34. Benakis C, Brea D, Caballero S, Faraco G, Moore J, Murphy M, Sita G, Racchumi G, Ling L, Pamer EG, et al. Commensal microbiota affects ischemic stroke outcome by regulating intestinal γδ T cells. *Nat Med* (2016) **22**:516–523. doi:10.1038/nm.4068

35. Singh V, Roth S, Llovera G, Sadler R, Garzetti D, Stecher B, Dichgans M, Liesz A. Microbiota Dysbiosis Controls the Neuroinflammatory Response after

Stroke. *J Neurosci* (2016) **36**:7428–7440. doi:10.1109/MSSC.2013.2278093

36. Fröhlich EE, Farzi A, Mayerhofer R, Reichmann F, Jačan A, Wagner B, Zinser E, Bordag N, Magnes C, Fröhlich E, et al. Cognitive impairment by antibiotic-induced gut dysbiosis: Analysis of gut microbiota-brain communication. *Brain Behav Immun* (2016) **56**:140–155. doi:10.1016/j.bbi.2016.02.020

37. Fetissov SO, Hamze Sinno M, Coëffier M, Bole-Feysot C, Ducrotté P, Hökfelt T, Déchelotte P. Autoantibodies against appetite-regulating peptide hormones and neuropeptides: Putative modulation by gut microflora. *Nutrition* (2008) **24**:348–359. doi:10.1016/j.nut.2007.12.006

38. Barrett E, Ross RP, O'Toole PW, Fitzgerald GF, Stanton C. γ-Aminobutyric acid production by culturable bacteria from the human intestine. *J Appl Microbiol* (2012) **113**:411–417. doi:10.1111/j.1365-2672.2012.05344.x

39. Forsythe P, Sudo N, Dinan T, Taylor VH, Bienenstock J. Mood and gut feelings. *Brain Behav Immun* (2010) **24**:9–16. doi:10.1016/j.bbi.2009.05.058

40. Lyte M. Probiotics function mechanistically as delivery vehicles for neuroactive compounds: Microbial endocrinology in the design and use of probiotics. *BioEssays* (2011) **33**:574–581. doi:10.1002/bies.201100024

41. Wang H, Yu M, Ochani M, Amella CA. Nicotinic acetylcholine receptor α7 subunit is an essential regulator of inflammation. *Nature* (2003) **421**:384–388. doi:10.1038/nature01339

42. Lisa E. Goehler, Su Mi Park, Noel Opitz, Mark Lyte and RPAG. Campylobacter jejuni infection increases anxiety-like behavior in the holeboard: possible anatomical substrates for viscerosensory modulation of exploratory behavior. *Brain Behav Immun* (2008) **22**:354–366.

43. Bravo JA, Forsythe P, Chew M V, Escaravage E, Savignac HM. Ingestion of

Lactobacillus strain regulates emotional. *PNSA* (2011) **108**:16050–16055. doi:10.1073/pnas.1102999108/-/DCSupplemental.www.pnas.org/cgi/doi/10.1073/pnas.1102999108

44. Bercik P, Park AJ, Sinclair D, Khoshdel A, Lu J, Huang X, Deng Y, Blennerhassett PA, Fahnestock M, Moine D, et al. The anxiolytic effect of Bifidobacterium longum NCC3001 involves vagal pathways for gut-brain communication. *Neurogastroenterol Motil* (2011) **23**:1132–1139. doi:10.1111/j.1365-2982.2011.01796.x

45. Round JL, Mazmanian SK. The gut microbiome shapes intestinal immune responses during health and disease. *Nat Rev Immunol* (2009) **9**:25. doi:10.1038/nri2515.The

46. Sadeghian S, Darvish S, Davoodi G, Salarifar M, Mahmoodian M, Fallah N, Karimi AA. The association of opium with coronary artery disease. *Eur J Prev Cardiol* (2007) **14**:715–717. doi:10.1097/HJR.0b013e328045c4e9

47. Hosseini SY, Safarinejad MR, Amini E, Hooshyar H. Opium consumption and risk of bladder cancer: A case-control analysis. *Urol Oncol Semin Orig Investig* (2010) **28**:610–616. doi:10.1016/j.urolonc.2008.10.016

48. Acharya C, Betrapally NS, Gillevet PM, Sterling RK, Akbarali H, White MB, Ganapathy D, Fagan A, Sikaroodi M, Bajaj JS. Chronic opioid use is associated with altered gut microbiota and predicts readmissions in patients with cirrhosis. *Aliment Pharmacol Ther* (2017) **45**:319–331. doi:10.1111/apt.13858

49. Cornford CS, Mason JM, Inns F. Deep vein thromboses in users of opioid drugs: Incidence, prevalence, and risk factors. *Br J Gen Pract* (2011) **61**:781–786. doi:10.3399/bjgp11X613115

50. Winkelman TNA, Chang VW, Binswanger IA. Health, Polysubstance Use, and Criminal Justice Involvement Among Adults With Varying Levels of Opioid Use. *JAMA Netw Open* (2018) **1**:e180558. doi:10.1001/jamanetworkopen.2018.0558

51. Downar J, Colman R, Horton R, Hernandez P, Rocker G. Opioids in COPD: A cause of death or a marker of illness severity? *Eur Respir J* (2016) **48**:1520–1521. doi:10.1183/13993003.01443-2016

52. Malekzadeh MM, Khademi H, Pourshams A, Etemadi A, Poustchi H, Bagheri M, Khoshnia M, Sohrabpour AA, Aliasgari A, Jafari E, et al. Opium use and risk of mortality from digestive diseases: A prospective cohort study. *Am J Gastroenterol* (2013) **108**:1757–1765. doi:10.1038/ajg.2013.336

53. Sabita Roy, Jana Ninkovic, Santanu Banerjee, Richard Charboneau S Das, Raini Dutta, Varvara Kirchner, Lisa Koodie, Jing Ma and JM. Opioid drug abuse and modulation of immune function: consequences in the susceptibility to opportunistic infections. *J neuroimmune pharmacol* (2011) **6**:442–465. doi:10.1007/s11481-011-9292-5

54. Cron DC, Englesbe MJ, Bolton CJ, Joseph MT, Carrier KL, Moser SE, Waljee JF, Hilliard PE, Kheterpal S, Brummett CM. Preoperative opioid use is independently associated with increased costs and worse outcomes after major abdominal surgery. *Ann Surg* (2017) **265**:695–701. doi:10.1097/SLA.0000000000001901

55. Zhang R, Meng J, Lian Q, Chen X, Bauman B, Chu H, Segura B, Roy S. Prescription opioids are associated with higher mortality in patients diagnosed with sepsis: A retrospective cohort study using electronic health records. *PLoS One* (2018) **13**:1–8. doi:10.1371/journal.pone.0190362

56. Padma Gulur, MD, Libby Williams, MPH, Sanjay Chaudhary, MHA, Katharine Koury B, and Michael Jaff D. Opioid Tolerance – A Predictor of Increased Length of Stay and Higher Readmission Rates. *Pain Physician* (2014)503–508.

57. Wang J, Barke RA, Charboneau R, Schwendener R, Roy S. Morphine Induces Defects in Early Response of Alveolar Macrophages to Streptococcus pneumoniae by Modulating TLR9-NF- B Signaling. *J Immunol* (2008) **180**:3594–3600. doi:10.4049/jimmunol.180.5.3594

58. Meng J, Banerjee S, Li D, Sindberg GM, Wang F, Ma J, Roy S. Opioid exacerbation of gram-positive sepsis, induced by gut microbial modulation, is rescued by IL-17A neutralization. *Sci Rep* (2015) **5**:1–17. doi:10.1038/srep10918

59. Meng J, Yu H, Ma J, Wang J, Banerjee S, Charboneau R, Barke RA, Roy S. Morphine Induces Bacterial Translocation in Mice by Compromising Intestinal Barrier Function in a TLR-Dependent Manner. *PLoS One* (2013) **8**: doi:10.1371/journal.pone.0054040

60. Wang F, Meng J, Zhang L, Johnson T, Chen C, Roy S. Morphine induces changes in the gut microbiome and metabolome in a morphine dependence model. *Sci Rep* (2018) **8**:1–15. doi:10.1038/s41598-018-21915-8

61. Szabo I, Rojavin M, Bussiere JL, Eisenstein TK, Adler MW, Rogers TJ. Suppression of peritoneal macrophage phagocytosis of Candida albicans by opioids. *J Pharmacol Exp Ther* (1993) **267**:703–706. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=8246144

62. Hilburger ME, Adler MW, Truant a L, Meissler JJ, Satishchandran V, Rogers

TJ, Eisenstein TK. Morphine induces sepsis in mice. *J Infect Dis* (1997) **176**:183–188. doi:doi:10.1086/514021

63. Lee K, Vuong HE, Nusbaum DJ, Hsiao EY, Evans CJ, Taylor AMW. The gut microbiota mediates reward and sensory responses associated with regimen-selective morphine dependence. *Neuropsychopharmacology* (2018)1. doi:10.1038/s41386-018-0211-9

64. Acharya C, Betrapally NS, Gillevet PM, Sterling RK, Akbarali H, White MB, Ganapathy D, Fagan A, Sikaroodi M, Bajaj JS. Chronic opioid use is associated with altered gut microbiota and predicts readmissions in patients with cirrhosis. *Aliment Pharmacol Ther* (2017) **45**:319–331. doi:10.1111/apt.13858

65. Zaborin A, Smith D, Garfield K, Quensen J, Shakhsheer B, Kade M, Tirrell M, Tiedje J, Gilbert JA, Zaborina O, et al. Membership and Behavior of Ultra-Low-Diversity Pathogen Critical Illness. *MBio* (2014) **5**:1–14. doi:10.1128/mBio.01361-14.Invited

66. Wood JD, Galligan JJ. Function of opioids in the enteric nervous system. *Neurogastroenterol Motil* (2004) **16**:17–28. doi:10.1111/j.1743-3150.2004.00554.x

67. Sobczak M, Sałaga M, Storr MA, Fichna J. Physiology, signaling, and pharmacology of opioid receptors and their ligands in the gastrointestinal tract: Current concepts and future perspectives. *J Gastroenterol* (2014) **49**:24–45. doi:10.1007/s00535-013-0753-x

68. Mischel RA, Dewey WL, Akbarali HI. Tolerance to Morphine-Induced Inhibition of TTX-R Sodium Channels in Dorsal Root Ganglia Neurons Is Modulated by Gut-Derived Mediators. *iScience* (2018) **2**:193–209.

doi:10.1016/j.isci.2018.03.003

69. Kang M, Mischel RA, Bhave S, Komla E, Cho A, Huang C, Dewey WL, Akbarali HI. The effect of gut microbiome on tolerance to morphine mediated antinociception in mice. *Sci Rep* (2017) **7**:1–17. doi:10.1038/srep42658

70. Lee K, Vuong HE, Nusbaum DJ, Hsiao EY, Evans CJ, Taylor AMW. The gut microbiota mediates reward and sensory responses associated with regimen-selective morphine dependence. *Neuropsychopharmacology* (2018) **43**:2606–2614. doi:10.1038/s41386-018-0211-9

71. Xu Y, Xie Z, Wang H, Shen Z, Guo Y, Gao Y. Bacterial Diversity of Intestinal Microbiota in Patients with Substance Use Disorders Revealed by 16S rRNA Gene Deep Sequencing. *Sci Rep* (2017) **7**:1–9. doi:10.1038/s41598-017-03706-9

72. Ferslew BC, Johnston CK, Tsakalozou E, Bridges AS, Paine MF, Jia W, Stewart PW, Barritt AS, Brouwer KLR. Altered morphine glucuronide and bile acid disposition in patients with nonalcoholic steatohepatitis. *Clin Pharmacol Ther* (2015) **97**:419–427. doi:10.1002/cpt.66

73. Dowell D, Haegerich TM CR. CDC Guideline for Prescribing Opioids for Chronic Pain — United States, 2016 Morbidity and Mortality Weekly Report. *Recomm Reports* (2016) **65**: doi:http://dx.doi.org/10.15585/mmwr.rr6501e1

74. Boyer EW. Management of Opioid Analgesic Overdose. *N Engl J Med* (2012) **367**:146–155. doi:10.1056/NEJMra1202561

75. Watkins LR, Shavit Y, Hutchinson MR, Maier SF, Rice KC, Grace PM. Exploring the Neuroimmunopharmacology of Opioids: An Integrative Review of Mechanisms of Central Immune Signaling and Their Implications for Opioid Analgesia. *Pharmacol Rev* (2011) **63**:772–810. doi:10.1124/pr.110.004135

76. Zhao CM, Guo RX, Hu F, Meng JL, Mo LQ, Chen PX, Liao XX, Cui Y, Feng JQ. Spinal MCP-1 contributes to the development of morphine antinociceptive tolerance in rats. *Am J Med Sci* (2012) **344**:473–479. doi:10.1097/MAJ.0b013e31826a82ce

77. Leduc-Pessah H, Weilinger NL, Fan CY, Burma NE, Thompson RJ, Trang T. Site-Specific Regulation of P2X7 Receptor Function in Microglia Gates Morphine Analgesic Tolerance. *J Neurosci* (2017) **37**:10154–10172. doi:10.1523/jneurosci.0852-17.2017

78. Liang Y, Chu H, Jiang Y, Yuan L. Morphine enhances IL-1β release through toll-like receptor 4-mediated endocytic pathway in microglia. *Purinergic Signal* (2016) **12**:637–645. doi:10.1007/s11302-016-9525-4

79. Parsadaniantz SM, Rivat C, Rostène W, Réaux-Le Goazigo A. Opioid and chemokine receptor crosstalk: A promising target for pain therapy? *Nat Rev Neurosci* (2015) **16**:69–78. doi:10.1038/nrn3858

80. Raghavendra V, Rutkowski MD, DeLeo JA. The Role of Spinal Neuroimmune Activation in Morphine Tolerance/Hyperalgesia in Neuropathic and Sham-Operated Rats. *J Neurosci* (2018) **22**:9980–9989. doi:10.1523/jneurosci.22-22-09980.2002

81. Liu D, Zhang X, Bu H, Shu B, Wang W, Luo A, Peng Y, Yang H, Tian X, Gao F, et al. Potential role of CXCL10/CXCR3 signaling in the development of morphine tolerance in periaqueductal gray. *Neuropeptides* (2017) **65**:120–127. doi:10.1016/j.npep.2017.07.004

82. Lin CP, Kang KH, Tu HJ, Wu MY, Lin TH, Liou HC, Sun WZ, Fu WM. CXCL12/CXCR4 Signaling Contributes to the Pathogenesis of Opioid Tolerance: A Translational Study. *Anesth Analg* (2017) **124**:972–979.

doi:10.1213/ANE.0000000000001480

83. BO Y, GUO G-Q, CHEN D-L, JIN H, LI Y-H, XU J-S. Complement factor C5a and C5a receptor contribute to morphine tolerance and withdrawal-induced hyperalgesia in rats. *Exp Ther Med* (2012) **4**:723–727. doi:10.3892/etm.2012.636

84. Berta T, Liu YC, Xu ZZ, Ji RR. Tissue plasminogen activator contributes to morphine tolerance and induces mechanical allodynia via astrocytic IL-1β and ERK signaling in the spinal cord of mice. *Neuroscience* (2013) **247**:376–385. doi:10.1016/j.neuroscience.2013.05.018

85. Nakamoto K, Kawasaki S, Kobori T, Fujita-Hamabe W, Mizoguchi H, Yamada K, Nabeshima T, Tokuyama S. Involvement of matrix metalloproteinase-9 in the development of morphine tolerance. *Eur J Pharmacol* (2012) **683**:86–92. doi:10.1016/j.ejphar.2012.03.006

86. Liu W, Wang CH, Cui Y, Mo LQ, Zhi JL, Sun SN, Wang YL, Yu HM, Zhao CM, Feng JQ, et al. Inhibition of neuronal nitric oxide synthase antagonizes morphine antinociceptive tolerance by decreasing activation of p38 MAPK in the spinal microglia. *Neurosci Lett* (2006) **410**:174–177. doi:10.1016/j.neulet.2006.08.091

87. Lin SL, Tsai RY, Tai YH, Cherng CH, Wu CT, Yeh CC, Wong CS. Ultra-low dose naloxone upregulates interleukin-10 expression and suppresses neuroinflammation in morphine-tolerant rat spinal cords. *Behav Brain Res* (2010) **207**:30–36. doi:10.1016/j.bbr.2009.09.034

88. Harada S, Nakamoto K, Tokuyama S. The involvement of midbrain astrocyte in the development of morphine tolerance. *Life Sci* (2013) **93**:573–578. doi:10.1016/j.lfs.2013.08.009

89. Song P, Zhao ZQ. The involvement of glial cells in the development of morphine tolerance. *Neurosci Res* (2001) **39**:281–286. doi:10.1016/S0168-0102(00)00226-1

90. Eidson LN, Murphy AZ. Blockade of Toll-Like Receptor 4 attenuates morphine tolerance and facilitates the pain relieving properties of morphine. *J Neurosci* (2013) **33**:15952–15963. doi:10.1523/jneurosci.1609-13.2013

91. Lewis SS, Hutchinson MR, Rezvani N, Loram LC, Maier SF, Rice KC, Watkins LR. Evidence that intrathecal morphine-3-glucuronide may cause pain enhancement via toll-like receptor 4/MD-2 and interleukin-1β. *Neuroscience* (2010) **165**:569–583. doi:10.1016/j.neuroscience.2009.10.011.Evidence

92. Qu J, Tao XY, Teng P, Zhang Y, Guo CL, Hu L, Qian YN, Jiang CY, Liu WT. Blocking ATP-sensitive potassium channel alleviates morphine tolerance by inhibiting HSP70-TLR4-NLRP3-mediated neuroinflammation. *J Neuroinflammation* (2017) **14**:1–17. doi:10.1186/s12974-017-0997-0

93. Wang X, Loram LC, Ramos K, de Jesus AJ, Thomas J, Cheng K, Reddy A, Somogyi AA, Hutchinson MR, Watkins LR, et al. Morphine activates neuroinflammation in a manner parallel to endotoxin. *Proc Natl Acad Sci* (2012) **109**:6325–6330. doi:10.1073/pnas.1200130109

94. Liang Liu, Janet K. Coller, Linda R. Watkins, Andrew A. Somogyi and MR, Hutchinson. Naloxone-precipitated morphine withdrawal behavior and brain IL-1β expression: comparison of different mouse strains. *Brain Behav Immun* (2012) **25**:1223–1232. doi:10.1016/j.bbi.2011.03.016.Naloxone-precipitated

95. Stokes JA, Corr M, Yaksh TL. Spinal Toll-like receptor signaling and nociceptive processing: Regulatory balance between TIRAP and TRIF cascades mediated by TNF and IFNβ. *Pain* (2013) **154**:733–742.

doi:10.1016/j.pain.2013.01.012

96. Na HS, Jo E-K, Park K, Cho I-H, Kim MS, Kim MA, Akira S, Kim JS, Lee S, Lee SJ, et al. A Critical Role of Toll-like Receptor 2 in Nerve Injury-induced Spinal Cord Glial Cell Activation and Pain Hypersensitivity. *J Biol Chem* (2007) **282**:14975–14983. doi:10.1074/jbc.m607277200

97. Marrow B, Secreted S, Protect C. Essential role of Toll-Like Receptor 2 in morphine-induced microglia activation in mice. *Neurosci Lett* (2014) **71**:3831–3840. doi:10.1158/0008-5472.CAN-10-4002.BONE

98. Li Y, Sun XL, Zhang Y, Huang JJ, Hanley G, Ferslew KE, Peng Y, Yin DL. Morphine promotes apoptosis via TLR2, and this is negatively regulated by β-arrestin 2. *Biochem Biophys Res Commun* (2009) **378**:857–861. doi:10.1016/j.bbrc.2008.12.001

99. Shafie A, Moradi F, Izadpanah E, Mokarizadeh A, Moloudi MR, Nikzaban M, Hassanzadeh K. Neuroprotection of donepezil against morphine-induced apoptosis is mediated through Toll-like receptors. *Eur J Pharmacol* (2015) **764**:292–297. doi:10.1016/j.ejphar.2015.07.027

100. Khademi H, Kamangar F, Brennan P, Malekzadeh R. Opioid Therapy and its Side Effects: A Review. *Arch Iran Med* (2016) **19**:870–876. doi:0161912/AIM.0010

101. Bailey CP, Connor M. Opioids: Cellular mechanisms of tolerance and physical dependence. *Curr Opin Pharmacol* (2005) **5**:60–68. doi:10.1016/j.coph.2004.08.012

102. Williams JT, Christie MJ, Manzoni O. Cellular and synaptic adaptations mediating opioid dependence. *Physiol Rev* (2001) **81**:299–343. doi:10.1016/0014-2999(92)90161-v

103. Stein C. Opioid Receptors. *Encycl Biol Chem Second Ed* (2013)354–358. doi:10.1016/B978-0-12-378630-2.00347-9

104. Hiroshi Ueda MU. Mechanisms underlying morphine analgesic tolerance and dependence. *Front Biosci* (2009) **14**:5260–5272.

105. Martini L, Whistler JL. The role of mu opioid receptor desensitization and endocytosis in morphine tolerance and dependence. *Curr Opin Neurobiol* (2007) **17**:556–564. doi:10.1016/j.conb.2007.10.004

106. S Tumati, WR Roeske, TM Largent-Milnes, TW Vanderah and EV. Intrathecal PKA-selective siRNA treatment blocks sustained morphine-mediated pain sensitization and antinociceptive tolerance in rats. *J Neurosci Methods* (2011) **199**:62–68. doi:10.1016/j.jneumeth.2011.04.036.

107. Chaves C, Remiao F, Cisternino S, Decleves X. Opioids and the Blood-Brain Barrier: A Dynamic Interaction with Consequences on Drug Disposition in Brain. *Curr Neuropharmacol* (2017) **15**:1156–1173. doi:10.2174/1570159x15666170504095823

108. Sharma HS, Sjöquist PO AS. Alterations in blood-brain barrier function and brain pathology by morphine in the rat. Neuroprotective effects of antioxidant H-290/51. *Acta Neurochir Suppl* (2010) **106**:61–66. doi:10.1007/BF00165138

109. Sharma HS, Sjöquist PO AS. Alterations in blood-brain barrier function and brain pathology by morphine in the rat. Neuroprotective effects of antioxidant H-290/51. *Acta Neurochir Suppl* **2010**:61–66. doi:10.1007/978-3-211-98811-4

110. Wickham RJ. Cancer Pain Management: Opioid Analgesics, Part 2. *J Adv Pr Oncol* (2017) **Vol 8**:588–607.

111. Cherny BN, Ripamonti C, Pereira J, Davis C, Fallon M, Mcquay H, Mercadante S. Morphine : An Evidence-Based Report. *Amercian Soc Clin*

Oncol (2001) **19**:2542–2554.

112. Volkow ND, McLellan AT. Opioid Abuse in Chronic Pain — Misconceptions and Mitigation Strategies. *N Engl J Med* (2016) **374**:1253–1263. doi:10.1056/NEJMra1507771

113. Jalal H, Buchanich J, Roberts MS, Balmert L, Zhang K, Burke DS. Dynamics of the drug overdose epidemic in the United States, 1979-2016. *Science (80-)* (2018) **1184**: doi:10.1126/science.aau1184

114. Dumas EO, Pollack GM. Opioid Tolerance Development: A Pharmacokinetic/Pharmacodynamic Perspective. *AAPS J* (2008) **10**: doi:10.1208/s12248-008-9056-1

115. Gilbert JA, Blaser MJ, Caporaso JG, Jansson JK, Lynch S V., Knight R. Current understanding of the human microbiome. *Nat Med* (2018) **24**:392–400. doi:10.1038/nm.4517

116. Fung TC, Olson CA, Hsiao EY. Interactions between the microbiota, immune and nervous systems in health and disease. *Nat Neurosci* (2017) **20**:145–155. doi:10.1038/nn.4476

117. Carvalho FA, Aitken JD, Vijay-Kumar M, Gewirtz AT. Toll-Like Receptor–Gut Microbiota Interactions: Perturb at Your Own Risk! *Annu Rev Physiol* (2012) **74**:177–198. doi:10.1146/annurev-physiol-020911-153330

118. Banerjee S, Sindberg G, Wang F, Meng J, Sharma U, Zhang L, Dauer P, Chen C, Dalluge J, Johnson T, et al. Opioid-induced gut microbial disruption and bile dysregulation leads to gut barrier compromise and sustained systemic inflammation. *Mucosal Immunol* (2016) **9**:1418–1428. doi:10.1038/mi.2016.9

119. Barengolts E, Green SJ, Eisenberg Y, Akbar A, Reddivari B, Layden BT, Dugas L, Chlipala G. Gut microbiota varies by opioid use, circulating leptin

and oxytocin in African American men with diabetes and high burden of chronic disease. *PLoS One* (2018) **13**:1–22. doi:10.1371/journal.pone.0194171

120. Kiraly DD, Walker DM, Calipari ES, Labonte B, Issler O, Pena CJ, Ribeiro EA, Russo SJ, Nestler EJ. Alterations of the host microbiome affect behavioral responses to cocaine. *Sci Rep* (2016) **6**:1–12. doi:10.1038/srep35455

121. Degirolamo C, Rainaldi S, Bovenga F, Murzilli S, Moschetta A. Microbiota modification with probiotics induces hepatic bile acid synthesis via downregulation of the Fxr-Fgf15 axis in mice. *Cell Rep* (2014) **7**:12–18. doi:10.1016/j.celrep.2014.02.032

122. Henderson-Redmond AN, Yuill MB, Lowe TE, Kline AM, Zee ML, Guindon J, Morgan DJ. Morphine-induced antinociception and reward in "humanized" mice expressing the mu opioid receptor A118G polymorphism. *Brain Res Bull* (2016) **123**:5–12. doi:10.1016/j.brainresbull.2015.10.007

123. Gohl DM, Vangay P, Garbe J, MacLean A, Hauge A, Becker A, Gould TJ, Clayton JB, Johnson TJ, Hunter R, et al. Systematic improvement of amplicon marker gene methods for increased accuracy in microbiome studies. *Nat Biotechnol* (2016) **34**:942–949. doi:10.1038/nbt.3601

124. Utente. Embnet_Journal_17_Vol1_Full.Pdf. (2015)1–3. doi:10.14806/ej.17.1.200

125. Jari Oksanen, F. Guillaume Blanchet, Michael Friendly, Roeland Kindt, Pierre Legendre, Dan McGlinn, Peter R. Minchin, R. B. O'Hara, Gavin L. Simpson, Peter Solymos, M. Henry H. Stevens ES and HW. CRAN - Package vegan. (2018) Available at: https://cran.r-project.org/web/packages/vegan/index.html

126. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. *Nat*

Methods (2016) **13**:581–583. doi:10.1038/nmeth.3869

127. Jiang W, Wu N, Wang X, Chi Y, Zhang Y, Qiu X, Hu Y, Li J, Liu Y. Dysbiosis gut microbiota associated with inflammation and impaired mucosal immune function in intestine of humans with non-alcoholic fatty liver disease. *Sci Rep* (2015) **5**:1–7. doi:10.1038/srep08096

128. Garidou L, Pomié C, Klopp P, Waget A, Charpentier J, Aloulou M, Giry A, Serino M, Stenman L, Lahtinen S, et al. The Gut Microbiota Regulates Intestinal CD4 T Cells Expressing RORγt and Controls Metabolic Disease. *Cell Metab* (2015) **22**:100–112. doi:10.1016/j.cmet.2015.06.001

129. Bäuerl C, Collado MC, Diaz Cuevas A, Viña J, Pérez Martínez G. Shifts in gut microbiota composition in an APP/PSS1 transgenic mouse model of Alzheimer's disease during lifespan. *Lett Appl Microbiol* (2018) **66**:464–471. doi:10.1111/lam.12882

130. Kaakoush NO. Insights into the Role of Erysipelotrichaceae in the Human Host. *Front Cell Infect Microbiol* (2015) **5**:1–4. doi:10.3389/fcimb.2015.00084

131. Reshef L, Kovacs A, Ofer A, Yahav L, Maharshak N, Keren N, Konikoff FM, Tulchinsky H, Gophna U, Dotan I. Pouch Inflammation Is Associated with a Decrease in Specific Bacterial Taxa. *Gastroenterology* (2015) **149**:718–727. doi:10.1053/j.gastro.2015.05.041

132. Scarpa M, Grillo A, Faggian D, Ruffolo C, Bonello E, D'Incà R, Scarpa M, Castagliuolo I, Angriman I. Relationship between mucosa-associated microbiota and inflammatory parameters in the ileal pouch after restorative proctocolectomy for ulcerative colitis. *Surgery* (2011) **150**:56–67. doi:10.1016/j.surg.2011.02.009

133. Lowe PP, Gyongyosi B, Satishchandran A, Iracheta-Vellve A, Ambade A,

Kodys K, Catalano D, Ward D V., Szabo G. Alcohol-related changes in the intestinal icrobiome influence neutrophil infiltration, inflammation and steatosis in early alcoholic hepatitis in mice. *PLoS One* (2017) **12**:1–16. doi:10.1371/journal.pone.0174544

134. Lee SM, Han HW, Yim SY. Beneficial effects of soy milk and fiber on high cholesterol diet-induced alteration of gut microbiota and inflammatory gene expression in rats. *Food Funct* (2015) **6**:492–500. doi:10.1039/c4fo00731j

135. Wang W, Zhao J, Gui W, Sun D, Dai H, Xiao L, Chu H, Du F, Zhu Q, Schnabl B, et al. Tauroursodeoxycholic acid inhibits intestinal inflammation and barrier disruption in mice with non-alcoholic fatty liver disease. *Br J Pharmacol* (2018) **175**:469–484. doi:10.1111/bph.14095

136. Thevaranjan N, Puchta A, Schulz C, Naidoo A, Szamosi JC, Verschoor CP, Loukov D, Schenck LP, Jury J, Foley KP, et al. Age-Associated Microbial Dysbiosis Promotes Intestinal Permeability, Systemic Inflammation, and Macrophage Dysfunction. *Cell Host Microbe* (2017) **21**:455-466.e4. doi:10.1016/j.chom.2017.03.002

137. Chen J, Chia N, Kalari KR, Yao JZ, Novotna M, Soldan MMP, Luckey DH, Marietta E V., Jeraldo PR, Chen X, et al. Multiple sclerosis patients have a distinct gut microbiota compared to healthy controls. *Sci Rep* (2016) **6**:1–10. doi:10.1038/srep28484

138. Martín-Hernández D, Caso JR, Bris ÁG, Maus SR, Madrigal JLM, García-Bueno B, MacDowell KS, Alou L, Gómez-Lus ML, Leza JC. Bacterial translocation affects intracellular neuroinflammatory pathways in a depression-like model in rats. *Neuropharmacology* (2016) **103**:122–133. doi:10.1016/j.neuropharm.2015.12.003

139. Liu W, Crott JW, Lyu L, Pfalzer AC, Li J, Choi SW, Yang Y, Mason JB, Liu Z. Diet-and genetically-induced obesity produces alterations in the microbiome, inflammation and Wnt pathway in the intestine of Apc +/1638N mice: Comparisons and contrasts. *J Cancer* (2016) **7**:1780–1790. doi:10.7150/jca.15792

140. Wang B, Xu H, Wei H, Zeng Z, Xu F. Oral administration of *Bifidobacterim bifidum* for modulating microflora, acid and bile resistance, and physiological indices in mice. *Can J Microbiol* (2015) **61**:155–163. doi:10.1139/cjm-2014-0694

141. Kobayashi Y, Sugahara H, Shimada K, Mitsuyama E, Kuhara T, Yasuoka A, Kondo T, Abe K, Xiao JZ. Therapeutic potential of Bifidobacterium breve strain A1 for preventing cognitive impairment in Alzheimer's disease. *Sci Rep* (2017) **7**:1–10. doi:10.1038/s41598-017-13368-2

142. Kang DJ, Betrapally NS, Ghosh SA, Sartor RB, Hylemon PB, Gillevet PM, Sanyal AJ, Heuman DM, Carl D, Zhou H, et al. Gut microbiota drive the development of neuroinflammatory response in cirrhosis in mice. *Hepatology* (2016) **64**:1232–1248. doi:10.1002/hep.28696

143. Mayer E a, Tillisch K, Gupta A, Mondello P, Mian M, Aloisi C, Famà F, Mondello S, Pitini V, Altamira-pa PNASEDE, et al. Gut/brain axis and the microbiota Emeran. *Nutr Cancer* (2015) **125**:463–479. doi:10.1172/JCI76304.Several

144. Fagundes CT, Teixeira MM, Costa V V., Amaral FA, Souza DG, Cunha TM, Ferreira SH, Nicoli JR, Cunha FQ, Cisalpino D, et al. Commensal microbiota is fundamental for the development of inflammatory pain. *Proc Natl Acad Sci* (2008) **105**:2193–2197. doi:10.1073/pnas.0711891105

145. Luczynski P, Neufeld KAMV, Oriach CS, Clarke G, Dinan TG, Cryan JF. Growing up in a bubble: Using germ-free animals to assess the influence of the gut microbiota on brain and behavior. *Int J Neuropsychopharmacol* (2016) **19**:1–17. doi:10.1093/ijnp/pyw020

146. Harach T, Marungruang N, Duthilleul N, Cheatham V, Mc Coy KD, Frisoni G, Neher JJ, Fåk F, Jucker M, Lasser T, et al. Reduction of Abeta amyloid pathology in APPPS1 transgenic mice in the absence of gut microbiota. *Sci Rep* (2017) **7**:46856. doi:10.1038/srep46856

147. Berer K, Mues M, Koutrolos M, AlRasbi Z, Boziki M, Johner C, Wekerle H, Krishnamoorthy G. Commensal microbiota and myelin autoantigen cooperate to trigger autoimmune demyelination. *Nature* (2011) **479**:538–541. doi:10.1038/nature10554

148. Mischel RA, Dewey WL, Akbarali HI, Mischel RA, Dewey WL, Akbarali HI. Tolerance to Morphine-Induced Inhibition of TTX-R Sodium Channels in Dorsal Root Ganglia Neurons Is Modulated by Gut-Derived Mediators Tolerance to Morphine-Induced Inhibition of TTX-R Sodium Channels in Dorsal Root Ganglia Neurons Is Modulated by Gut-Der. *iScience* (2018) **2**:1–17. doi:10.1016/j.isci.2018.03.003

149. Ma W, Zheng W-H, Powell K, Jhamandas K, Quirion R. Chronic morphine exposure increases the phosphorylation of MAP kinases and the transcription factor CREB in dorsal root ganglion neurons: An in vitro and in vivo study. *Eur J Neurosci* (2001) **14**:1091–1104. doi:10.1046/j.0953-816X.2001.01731.x

150. Shavit Y, Wolf G, Goshen I, Livshits D, Yirmiya R. Interleukin-1 antagonizes morphine analgesia and underlies morphine tolerance. *Pain* (2005) **115**:50–59. doi:10.1016/j.pain.2005.02.003

151. Shen CH, Tsai RY, Wong CS. Role of neuroinflammation in morphine tolerance: Effect of tumor necrosis factor-α. *Acta Anaesthesiol Taiwanica* (2012) **50**:178–182. doi:10.1016/j.aat.2012.12.004

152. DeLeo JA, Tanga FY, Tawfik VL. Neuroimmune Activation and Neuroinflammation in Chronic Pain and Opioid Tolerance/Hyperalgesia. *Neuroscientist* (2004) **10**:40–52. doi:10.1177/1073858403259950

153. Martin D, Watkins LR, Hutchinson MR, Maier SF, Somogyi AA, Poole S, Judd CM, Coats BD, Wieseler JL, Jekich BM, et al. Proinflammatory cytokines oppose opioid-induced acute and chronic analgesia. *Brain Behav Immun* (2008) **22**:1178–1189. doi:10.1016/j.bbi.2008.05.004

154. Ian N. Johnston, Erin D. Milligan, Julie Wieseler-Frank, Matthew G. Frank, Varlin Zapata JC, Stephen Langer, David Martin, Paula Green, M. Fleshner, Leslie Leinwand, Steven F. Maier and LRW. A Role for Proinflammatory Cytokines and Fractalkine in Analgesia, Tolerance, and Subsequent Pain Facilitation Induced by Chronic Intrathecal Morphine. *J Neurosci* (2004) **24**:7353–7365. doi:10.1523/JNEUROSCI.1850-04.2004

155. Roeckel LA, Le Coz GM, Gavériaux-Ruff C, Simonin F. Opioid-induced hyperalgesia: Cellular and molecular mechanisms. *Neuroscience* (2016) **338**:160–182. doi:10.1016/j.neuroscience.2016.06.029

156. De Guglielmo G, Kallupi M, Scuppa G, Stopponi S, Demopulos G, Gaitanaris G, Ciccocioppo R. Analgesic tolerance to morphine is regulated by PPAR. *Br J Pharmacol* (2014) **171**:5407–5416. doi:10.1111/bph.12851

157. Han Y, Jiang C, Tang J, Wang C, Wu P, Zhang G, Liu W, Jamangulova N, Wu X, Song X. Resveratrol reduces morphine tolerance by inhibiting microglial activation via AMPK signalling. *Eur J Pain (United Kingdom)* (2014)

18:1458–1470. doi:10.1002/ejp.511

158. Cui Y, Liao XX, Liu W, Guo RX, Wu ZZ, Zhao CM, Chen PX, Feng JQ. A novel role of minocycline: Attenuating morphine antinociceptive tolerance by inhibition of p38 MAPK in the activated spinal microglia. *Brain Behav Immun* (2008) **22**:114–123. doi:10.1016/j.bbi.2007.07.014

159. Khan MI, Ostadhadi S, Mumtaz F, Momeny M, Moghaddaskho F, Hassanipour M, Ejtemaei-Mehr S, Dehpour AR. Thalidomide attenuates the development and expression of antinociceptive tolerance to μ-opioid agonist morphine through L-arginine-iNOS and nitric oxide pathway. *Biomed Pharmacother* (2017) **85**:493–502. doi:10.1016/j.biopha.2016.11.056

160. Seifert HA, Benedek G, Nguyen H, Gerstner G, Zhang Y, Kent G. Antibiotics protect against EAE by increasing regulatory and anti-inflammatory cells. *Metab Brain Dis* (2018) **33**:1599–1607.

161. Santa-Cecília FV, Leite CA, Del-Bel E, Raisman-Vozari R. The Neuroprotective Effect of Doxycycline on Neurodegenerative Diseases. *Neurotox Res* (2019) **35**:981–986. doi:10.1007/s12640-019-00015-z

162. Iracheta-Vellve A, Gyongyosi B, Ambade A, Cho Y, Lowe PP, Satishchandran A, Szabo G. Reduced gut microbiome protects from alcohol-induced neuroinflammation and alters intestinal and brain inflammasome expression. *J Neuroinflammation* (2018) **15**:1–12. doi:10.1186/s12974-018-1328-9

163. Dutta R, Krishnan A, Meng J, Das S, Ma J, Banerjee S, Wang J, Charboneau R, Prakash O, Barke RA, et al. Morphine Modulation of Toll-Like Receptors in Microglial Cells Potentiates Neuropathogenesis in a HIV-1 Model of Coinfection with Pneumococcal Pneumoniae. *J Neurosci* (2012) **32**:9917–9930. doi:10.1523/jneurosci.0870-12.2012

164. Liu R, Kang JD, Sartor RB, Sikaroodi M, Fagan A, Gavis EA, Zhou H, Hylemon PB, Herzog JW, Li X, et al. Neuroinflammation in Murine Cirrhosis is Dependent on the Gut Microbiome and is Attenuated by Fecal Transplant. *Hepatology* (2019)0–3. doi:10.1002/hep.30827

165. Brandt LJ, Aroniadis OC. An overview of fecal microbiota transplantation: techniques, indications, and outcomes. *Gastrointest Endosc* (2013) **78**:240–249. doi:10.1016/j.gie.2013.03.1329

166. Sarkar A, Mandal S. Bifidobacteria—Insight into clinical outcomes and mechanisms of its probiotic action. *Microbiol Res* (2016) **192**:159–171. doi:10.1016/j.micres.2016.07.001

167. Salehipour Z, Haghmorad D, Sankian M, Rastin M, Nosratabadi R, Soltan Dallal MM, Tabasi N, Khazaee M, Nasiraii LR, Mahmoudi M. Bifidobacterium animalis in combination with human origin of Lactobacillus plantarum ameliorate neuroinflammation in experimental model of multiple sclerosis by altering CD4+ T cell subset balance. *Biomed Pharmacother* (2017) **95**:1535–1548. doi:10.1016/j.biopha.2017.08.117

168. Carroll NM, Jaeger EEM, Choudhury S, Dunlop AAS, Matheson MM, Adamson P, Okhravi N, Lightman S. Detection of and discrimination between gram-positive and gram-negative bacteria in intraocular samples by using nested PCR. *J Clin Microbiol* (2000) **38**:1753–1757.

169. Rothhammer V, Mascanfroni ID, Bunse L, Takenaka MC, Jessica E, Mayo L, Chao C, Patel B, Yan R, Blain M, et al. Type I interferons and microbial metabolites of tryptophan modulate astrocyte activity and CNS inflammation via the aryl hydrocarbon receptor. *Nat Med* (2016) **22**:586–597. doi:10.1038/nm.4106.Type

170. Erny D, De Angelis ALH, Jaitin D, Wieghofer P, Staszewski O, David E, Keren-Shaul H, Mahlakoiv T, Jakobshagen K, Buch T, et al. Host microbiota constantly control maturation and function of microglia in the CNS. *Nat Neurosci* (2015) **18**:965–977. doi:10.1038/nn.4030

171. Ogbonnaya ES, Clarke G, Shanahan F, Dinan TG, Cryan JF, O'Leary OF. Adult Hippocampal Neurogenesis Is Regulated by the Microbiome. *Biol Psychiatry* (2015) **78**:e7–e9. doi:10.1016/j.biopsych.2014.12.023

172. Bortolotto V, Grilli M. Opiate analgesics as negative modulators of adult hippocampal neurogenesis: Potential implications in clinical practice. *Front Pharmacol* (2017) **8**:1–7. doi:10.3389/fphar.2017.00254

173. Neufeld KM, Kang N, Bienenstock J, Foster JA. Reduced anxiety-like behavior and central neurochemical change in germ-free mice. *Neurogastroenterol Motil* (2011) **23**:255–265. doi:10.1111/j.1365-2982.2010.01620.x

174. Sudo N, Chida Y, Aiba Y, Sonoda J, Oyama N, Yu XN, Kubo C, Koga Y. Postnatal microbial colonization programs the hypothalamic-pituitary-adrenal system for stress response in mice. *J Physiol* (2004) **558**:263–275. doi:10.1113/jphysiol.2004.063388

175. Zhao YL, Chen SR, Chen H, Pan HL. Chronic opioid potentiates presynaptic but impairs postsynaptic N-methyl-D-aspartic acid receptor activity in spinal cords: Implications for opioid hyperalgesia and tolerance. *J Biol Chem* (2012) **287**:25073–25085. doi:10.1074/jbc.M112.378737

176. Hörmann N, Brandão I, Jäckel S, Ens N, Lillich M, Walter U, Reinhardt C. Gut microbial colonization orchestrates TLR2 expression, signaling and epithelial proliferation in the small intestinal mucosa. *PLoS One* (2014) **9**:2–12.

doi:10.1371/journal.pone.0113080

177. Brenchley JM, Douek DC. Microbial Translocation Across the GI Tract. *Annu Rev Immunol* (2012) **30**:149–173. doi:10.1146/annurev-immunol-020711-075001

178. Kelly JR, Kennedy PJ, Cryan JF, Dinan TG, Clarke G, Hyland NP. Breaking down the barriers: the gut microbiome, intestinal permeability and stress-related psychiatric disorders. *Front Cell Neurosci* (2015) **9**:392. doi:10.3389/fncel.2015.00392

179. Di Felice G, Barletta B, Butteroni C, Corinti S, Tinghino R, Colombo P, Boirivant M. Use of probiotic bacteria for prevention and therapy of allergic diseases: studies in mouse model of allergic sensitization. *J Clin Gastroenterol* (2008) **42 Suppl 3**:S130-2. doi:10.1097/MCG.0b013e318169c463

180. Akbari E, Asemi Z, Kakhaki RD, Bahmani F, Kouchaki E, Tamtaji OR, Hamidi GA, Salami M. Effect of probiotic supplementation on cognitive function and metabolic status in Alzheimer's disease: A randomized, double-blind and controlled trial. *Front Aging Neurosci* (2016) **8**: doi:10.3389/fnagi.2016.00256

181. Reis DJ, Ilardi SS, Punt SEW. The anxiolytic effect of probiotics: A systematic review and meta-analysis of the clinical and preclinical literature. *PLoS One* (2018) **13**:1–25. doi:10.1371/journal.pone.0199041

182. Wallace CJK, Milev R. The effects of probiotics on depressive symptoms in humans: A systematic review. *Ann Gen Psychiatry* (2017) **16**:1–10. doi:10.1186/s12991-017-0138-2

183. Zareie M, Johnson-Henry K, Jury J, Yang PC, Ngan BY, McKay DM, Soderholm JD, Perdue MH, Sherman PM. Probiotics prevent bacterial

translocation and improve intestinal barrier function in rats following chronic psychological stress. *Gut* (2006) **55**:1553–1560. doi:10.1136/gut.2005.080739

184. Rousseaux C, Thuru X, Gelot A, Barnich N, Neut C, Dubuquoy L, Dubuquoy C, Merour E, Geboes K, Chamaillard M, et al. Lactobacillus acidophilus modulates intestinal pain and induces opioid and cannabinoid receptors. *Nat Med* (2007) **13**:35–37. doi:10.1038/nm1521

185. Distrutti E, O'Reilly JA, McDonald C, Cipriani S, Renga B, Lynch MA, Fiorucci S. Modulation of intestinal microbiota by the probiotic VSL#3 resets brain gene expression and ameliorates the age-related deficit in LTP. *PLoS One* (2014) **9**: doi:10.1371/journal.pone.0106503

186. Eidson LN, Murphy AZ. Persistent peripheral inflammation attenuates morphine-induced periaqueductal gray glial cell activation and analgesic tolerance in the male rat. *J Pain* (2013) **14**:393–404. doi:10.1016/j.jpain.2012.12.010

187. Zhang Y, Li H, Li Y, Sun X, Zhu M, Hanley G, LeSage G, Yin D. Essential role of toll-like receptor 2 in morphine-induced microglia activation in mice. *Neurosci Lett* (2011) **489**:43–47. doi:10.1016/j.neulet.2010.11.063

188. Bravo JA, Forsythe P, Chew M V, Escaravage E, Savignac HM, Dinan TG, Bienenstock J, Cryan JF. Ingestion of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. *Proc Natl Acad Sci U S A* (2011) **108**:16050–5. doi:10.1073/pnas.1102999108

189. Bercik P, Verdu EF, Foster JA, MacRi J, Potter M, Huang X, Malinowski P, Jackson W, Blennerhassett P, Neufeld KA, et al. Chronic gastrointestinal inflammation induces anxiety-like behavior and alters central nervous system

biochemistry in mice. *Gastroenterology* (2010) **139**:2102-2112.e1. doi:10.1053/j.gastro.2010.06.063

190. Dunne A, Neill LAJO. The Interleukin-1 Receptor / Toll-Like Receptor Superfamily. (2003)

191. Heinrich PC, Behrmann I, Haan S, Hermanns HM, Uller-newen GM. Principles of IL 6 type cytokine signaling and its regulating. *Biochem j* (2003) **374**:1–20. Available at: https://sci-hub.tw/10.1042/bj20030407

192. Qiu Z, Parsons K, Gruol D. Interleukin-6 selectively enhances the intracellular calcium response to NMDA in developing CNS neurons. *J Neurosci* (1995) **15**:6688–6699. doi:10.1523/jneurosci.15-10-06688.1995

193. Grace PM, Hutchinson MR, Bishop A, Somogyi AA, Mayrhofer G, Rolan PE. Adoptive transfer of peripheral immune cells potentiates allodynia in a graded chronic constriction injury model of neuropathic pain. *Brain Behav Immun* (2011) **25**:503–513. doi:10.1016/j.bbi.2010.11.018

194. Corder G, Tawfik VL, Wang D, Sypek EI, Low SA, Dickinson JR, Sotoudeh C, Clark JD, Barres BA, Bohlen CJ, et al. Loss of μ opioid receptor signaling in nociceptors, but not microglia, abrogates morphine tolerance without disrupting analgesia. *Nat Med* (2017) **23**:164–173. doi:10.1038/nm.4262

195. Li H, Tao R, Wang J, Xia L. Upregulation of miR-375 level ameliorates morphine analgesic tolerance in mouse dorsal root ganglia by inhibiting the JAK2/STAT3 pathway. *J Pain Res* (2017) **10**:1279–1287. doi:10.2147/JPR.S125264

196. Zhang Y, Wang K, Lin M, Li Q, Hong Y. Inhibition of morphine tolerance by MrgC receptor via modulation of interleukin-1β and matrix metalloproteinase 9 in dorsal root ganglia in rats. *Eur J Pharmacol* (2017) **815**:10–17.

doi:10.1016/j.ejphar.2017.10.011

197. Tabish Hussain, Hyunsuk Kil, Bharathi Hattiangady, Jaeho Lee, Maheedhar Kodali, Bing Shuai, Sahithi Attaluri, Yoko Takata, Jianjun Shen, Martin C. Abba, Asok K. Shetty CMA. Wwox deletion leads to reduced GABA-ergic inhibitory interneuron numbers and activation of microglia and astrocytes in mouse hippocampus. *Neurobiol Dis* (2019) **121**:163–176. doi:10.22201/fq.18708404e.2004.3.66178

198. Parkhurst CN, Yang G, Ninan I, Savas JN, Yates JR, Lafaille JJ, Hempstead BL, Littman DR, Gan WB. Microglia promote learning-dependent synapse formation through brain-derived neurotrophic factor. *Cell* (2013) **155**:1596–1609. doi:10.1016/j.cell.2013.11.030

199. Tien AC, Tsai HH, Molofsky A V., McMahon M, Foo LC, Kaul A, Dougherty JD, Heintz N, Gutmann DH, Barres BA, et al. Regulated temporal-spatial astrocyte precursor cell proliferation involves BRAF signalling in mammalian spinal cord. *Dev* (2012) **139**:2477–2487. doi:10.1242/dev.077214

200. Ridlon JM, Kang DJ, Hylemon PB, Bajaj JS. Bile Acids and the Gut Microbiome Introduction: The Human Gut Microbiome. (2015) **30**:332–338. doi:10.1097/MOG.0000000000000057

201. Zhao Q, Elson CO. Adaptive immune education by gut microbiota antigens. *Immunology* (2018) **154**:28–37. doi:10.1111/imm.12896

202. Ostaff MJ, Stange EF, Wehkamp J. Antimicrobial peptides and gut microbiota in homeostasis and pathology. *EMBO Mol Med* (2013) **5**:1465–1483. doi:10.1002/emmm.201201773

203. Roy S, Ninkovic J, Banerjee S, Charboneau RG, Das S, Dutta R, Kirchner VA, Koodie L, Ma J, Meng J, et al. Opioid drug abuse and modulation of immune

function: Consequences in the susceptibility to opportunistic infections. *J Neuroimmune Pharmacol* (2011) **6**:442–465. doi:10.1007/s11481-011-9292-5

204. Vital N, Reports S. National Vital Statistics Reports Drugs Most Frequently Involved in Drug Overdose. (2018) **67**:

205. Meckel KR, Kiraly DD. A potential role for the gut microbiome in substance use disorders. *Psychopharmacology (Berl)* (2019) doi:10.1007/s00213-019-05232-0

APPENDICES

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